J. Microbiol. Biotechnol. (2018), 28(4), 645–651
https://doi.org/10.4014/jmb.1712.12043

jmb

Alternative Carcinogenicity Screening Assay Using Colon Cancer Stem Cells: A Quantitative PCR (qPCR)-Based Prediction System for Colon Carcinogenesis

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Received: December 21, 2017
Revised: February 14, 2018
Accepted: February 18, 2018

First published online
March 15, 2018

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Supplementary data for this paper are available on-line only at http://jmb.or.kr.

pISSN 1017-7825, eISSN 1738-8872

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The carcinogenicity of chemicals in the environment is a major concern. Recently, numerous studies have attempted to develop methods for predicting carcinogenicity, including rodent and cell-based approaches. However, rodent carcinogenicity tests for evaluating the carcinogenic potential of a chemical to humans are time-consuming and costly. This study focused on the development of an alternative method for predicting carcinogenicity using quantitative PCR (qPCR) and colon cancer stem cells. A toxicogenomic method, mRNA profiling, is useful for predicting carcinogenicity. Using microarray analysis, we optimized 16 predictive gene sets from five carcinogens (azoxymethane, 3,2′-dimethyl-4-aminobiphenyl, *N*-ethyl-*n*-nitrosourea, metronidazole, 4-(*n*-methyl-*n*-nitrosamino)-1-(3-pyridyl)-1-butanone) used to treat colon cancer stem cell samples. The 16 genes were evaluated by qPCR using 23 positive and negative carcinogens in colon cancer stem cells. Among them, six genes could differentiate between positive and negative carcinogens with a p-value of ≤ 0.05. Our qPCR-based prediction system for colon carcinogenesis using colon cancer stem cells is cost- and time-efficient. Thus, this qPCR-based prediction system is an alternative to in vivo carcinogenicity screening assays.

Keywords: Microarray, quantitative PCR, colon cancer, cancer stem cell, carcinogenicity

Introduction

Despite great efforts to defeat cancer, it remains a major life-threatening disease and its incidence and death rates continue to increase [1]. Colorectal cancer is a leading cause of cancer mortality, causing 49,190 deaths in the United States in 2016 [1]. It has been reported that cancer stem cells are involved in cancer development and maintaining malignant lesions [2]. However, the precise mechanism of cancer stem cells in the carcinogenesis of colon cancer remains unclear. Additionally, the causes of cancer are diverse, and there is substantial evidence that environmental chemicals as well as inherited genetic factors play a major role in carcinogenesis [3]. Thus, various approaches have been developed for detecting the carcinogenicity of environmental pollutants and studying their precise mechanisms in carcinogenesis. In the last few decades, carcinogenic properties have been measured using long-term rodent bioassays. These time-consuming, costly, and inefficient tests involve the killing of numerous animals. However, their accuracy for detecting carcinogenicity to humans is only 60% [4]. Additionally, restrictive legislation against animal testing is increasing [5]. Besides this, the relevance of in vivo toxicity assays in rodents for humans remains a challenge, as its accuracy is approximately around 80% [6]. Taken together, alternative non-animal,

cost-effective tests are urgently needed. Transcriptomics, a method for global gene expression profiling, is considered an alternative to animal testing and has been applied to numerous fields of study, including the exploration of biomarkers, toxicity mechanism studies, and prediction of toxicity [7]. As reported previously, the first event in the response to chemical exposure is alteration of gene expression, resulting in phenotypic changes [8]. However, microarray analysis requires costly specialized equipment and bioinformatics approaches for data analysis, limiting its versatility. Therefore, we developed a simple and cost-effective alternative carcinogenicity screening assay to microarray-based assays. In this study, we established an alternative carcinogenicity screening assay involving a quantitative PCR (qPCR)-based prediction system for colon carcinogenesis using colon cancer stem cells.

Materials and Methods

Cell Culture and Chemicals

The human colon cancer cell line HCT116 was obtained from American Type Culture Collection (Manassas, VA, USA). HCT116 cells were cultured in DMEM medium (Hyclone, USA) supplemented with heat-inactivated 10% (v/v) fetal bovine serum (Sigma-Aldrich, USA). The cells were incubated at 37°C in a humidified incubator containing 5% CO_2. For cancer stem cells, 1,000 cells/ml were seeded on ultra-low attachment plates (Corning, Inc., USA) and cultured as described previously. The compounds that were used in this study are described in Table 1.

Cell viability Assays

Cell viability was quantified using a cell counting kit-8 (CCK-8) assay (Promega, USA). Cells were seeded at 5,000 cells per well into 96-well culture plates containing 100 μl of DMEM supplemented with 10% fetal bovine serum, 100 μg/ml penicillin, and 0.25 μg/ml streptomycin and cultured overnight. After 20 h of incubation, various concentrations of chemicals were added to the wells followed by incubation for an additional 72 h. Cell viability was analyzed by performing the CCK-8 assay according to the manufacturer's instructions. Optical density was measured at 450 nm using a microplate reader (Apollo LB 9110; Berthold Technologies GmbH, Germany).

Sphere Formation

HCT116 colon cancer cells were counted and plated in low-attachment plates (Corning, USA) at a constant density of 2,000 viable cells per milliliter. Cells were grown in DMEM/F12 medium (Invitrogen, USA) containing 1% fetal bovine serum, which was supplemented with 20 ng/ml epidermal growth factor (Sigma, USA), 10 ng/ml basic fibroblast growth factor (Sigma, USA), 1%

Table 1. List of 18 genotoxic carcinogen (GC) and 5 non-carcinogen (NC) compounds.

Compound class	Chemical name	CAS number	Source	Working Conc.
GC 1	Azoxymethane (AOM)	25843-45-2	Sigma	160 μg/ml
GC 2	Metrinidazole (MNZ)	443-48-1	Sigma	160 μg/ml
GC 3	4-(*n*-Methyl-*n*-nitrosamino)-1-(3-pyridyl)-1-butanone (NKK)	64091-91-4	Sigma	100 μg/ml
GC 4	*N*-Ethyl-*n*-nitrosourea (ENU)	759-73-9	Sigma	2.5 μg/ml
GC 5	3,2′-Dimethyl-4-aminobiphenyl (DMAB)	58109-32-3	SCBT	400 μg/ml
GC 6	2,4-Diaminotoluene	95-80-7	Sigma	160 μg/ml
GC 7	Furan	110-00-9	Sigma	800 μg/ml
GC 8	Quinoline (QN)	91-22-5	Sigma	16 μg/ml
GC 9	4-Nitroquinoline-1-oxide (4-NQO)	56-57-5	Sigma	10 ng/ml
GC 10	7,12-Dimethylbenz[a]anthracene (DMBA)	57-97-6	Sigma	16 μg/ml
GC 11	Mitomycin C (MMC)	1950-07-07	Sigma	10 ng/ml
GC 12	Benzo[a]pyrene (BaP)	50-32-8	Sigma	200 μg/ml
GC 13	2-Nitropropane (2-NP)	79-46-9	Sigma	200 μg/ml
GC 14	1,2-Dimethylhydrazine (SDMH)	306-37-6	Sigma	64 μg/ml
GC 15	*O*-Nitroanisole	91-23-6	Sigma	40 μg/ml
GC 16	Dextran sulfate sodium (DSS)	9011-18-1	Sigma	400 μg/ml
GC 17	PMA	16561-29-8	Sigma	50 ng/ml
GC 18	Paclitaxel (PTX)	33069-62-4	Sigma	10 ng/ml
NC 19	ε-Caprolactam (CAP)	105-60-2	Sigma	400 μg/ml
NC 20	D-Mannitol (MTL)	69-65-8	Sigma	400 μg/ml
NC 21	Lithocholic acid (LCA)	434-13-9	Sigma	40 μg/ml
NC 22	3-Chloro-*p*-toluidine (CPT)	95-74-9	SCBT	4 μg/ml
NC 23	Sodium benzoate (SB)	532-32-1	Sigma	640 μg/ml

N2 (Stemcell Technologies, Inc., Canada), and 5% B27 (Gibco, USA) for 7 days.

Microarray

To identify the genes regulated by azoxymethane (AOM), *N*-ethyl-*n*-nitrosourea (ENU), metrinidazole (MNZ), 4-(*n*-methyl-*n*-nitrosamino)-1-(3-pyridyl)-1-butanone (NKK), and 3,2′-dimethyl-4-aminobiphenyl (DMAB) in cancer stem cells, we performed microarray analysis. Total RNA was extracted using the Ribospin Kit (GeneAll, Korea) according to the manufacturer's instructions. The RNA quality was measured with an Agilent 2100 Bioanalyzer using the RNA 6000 Nano Chip (Agilent Technologies, USA), and the RNA quantity was measured with an ND-1000 Spectrophotometer (NanoDrop Technologies, Inc., USA). We used 300 ng of each RNA sample as input in the Affymetrix procedure according to the manufacturer's instructions. Total RNA from each sample was reverse-transcribed into cDNA. The cDNA was fragmented, end-labeled in a terminal transferase reaction, and ligated to a biotinylated dideoxynucleotide. Fragmented end-labeled cDNA was then hybridized to GeneChip Human Gene 1.0 ST arrays following the manufacturer's instructions. After 16 h of hybridization, the chips were stained and washed using the GeneChip Fluidics Station 450 (Affymetrix, USA), and scanned on the GeneChip Array scanner 3000 7G (Affymetrix, USA). An Affymetrix GeneChip Human Gene 1.0 ST Array was used to analyze the transcriptional profiles of the six samples, untreated HCT116 cancer stem cells, and carcinogen-treated HCT116 cancer stem cells. The Affymetrix array contains 764,885 25-mer oligonucleotide probes covering 28,869 human genes. Affymetrix analysis was conducted using the following steps: image acquisition, data extraction, normalization, differentially expressed gene selection, and functional grouping. Robust multi-array average was used for normalization. The Web-based tool DAVID (the Database for Annotation, Visualization, and Integrated Discovery) was used to identify the biological functions of differentially expressed genes. Next, these genes were categorized on the basis of gene function in the Gene Ontology and KEGG Pathway databases (http://david.abcc.ncifcrf.gov/home.jsp).

Gene Network with Compound-Mediated Signaling Utilizing the GeneMANIA Database and GIANT

Datasets, including coexpression, physical interactions, pathway, and genetic interactions, were collected from GeneMANIA. The five dataset-modulated carcinogens were produced from the GeneMANIA database (http://www.genemania.org). GIANT (Genome-scale Integrated Analysis of gene Networks in Tissues) was used for tissue-specific pathway analysis. The dataset is available at http://giant.princeton.edu/.

Reverse Transcription-PCR

Cells were lysed in 1 ml of easy-BLUE Total RNA Extraction Kit reagent (iNtRON Biotechnology, Korea) and RNA was isolated according to the manufacturer's instructions. Oligo (dT)-primed RNA (5 μg) was reverse-transcribed using M-MuLV reverse transcriptase (New England Biolabs, USA). The quantitative reverse transcription polymerase chain reaction (qRT-PCR) was performed using Rotor-Gene 6000 series software 1.7 (Qiagen, Germany) and the SensiFAST SYBR NO-ROX Kit (BIOLINE, UK) with the following primer sets: S100A7A (F: 5′-TTCACAAAT ACACCGGACGT-3′, R: 5′-CGAGGTAATGTATGCCCTTT-3′); ENO1 (F: 5′-TTCACAAATACACCGGACGT-3′, R: 5′-GCAGGC GCAATAGTTTTATT-3′); GOLGA7B (F: 5′-TTATCCAGAGAG ACTACAGC-3′, R: 5′-TTTTAGGCA ACTCCCAGAAG -3′); PGA5 (F: 5′-GACTTCCTGAAGAAGCACAA-3′, R: 5′-CATATCCAGGTA GTTCTCCA-3′); SCT (F: 5′-GACGCAGAGAACAGCATG-3′, R: 5′-CAGCTGGTTCTGAAACCAT-3′); MS4A4E (F: 5′-TTCTGA TTGCCTTGATGAGC-3′, R: 5′-TAAGGATACATCACTGACCC-3′); H3F3C (F: 5′-CAAGCAGACTGCTCGTAAAT-3′, R: 5′-GGTCGA CTTCTGATAACGAC-3′); KRTAP4-6 (F: 5′-CCTCTTGCTGTG AATCCAG-3′, R: 5′-GTGGAAATGACACAGGTTGG-3′); ZNF844 (F: 5′-CAGAGAAGTGATGCAGGAAA-3′, R: 5′-AAAGTAGTT GAGAGAGAGGG-3′); CDKL4 (F: 5′-CAGAAACAAAACCTC TGGAC-3′, R: 5′-CTCGATGAGGTTCACAAGAT-3′); RNPS1 (F: 5′-TGAAAAGGAGAGGAAAAGGC-3′, R: 5′-CACGGGCATGTC AATCATTT-3′); HIST1H2BH (F: 5′-GAAGAAGGATGGCAA GAAGC-3′, R: 5′-GGAATTCATGATCCCCATGG-3′); RPS12 (F: 5′-TCTTTGTGTGCTTGCATCCA-3′, R: 5′-TTACAAAGGCCTACC CATTC-3′); C6orf47 (F: 5′-CATCCCAAGACTAAGGACTC-3′, R: 5′-CCACTTGAGGGAATCCATTC-3′); CPA4 (F: 5′-GGGACC AAGTTTTGAGGATT-3′, R: 5′-GGAGGGAGATTTCCAGAAAT-3′); PLCXD1 (F: 5′-GACACACTCACGGAAATCTC-3′, R: 5′-ATGTTC TTGATACAGGCGAC-3′); and GAPDH (F: 5′-TGGGCTACA CTGAGCACCAG-3′, R: 5′-GGGTGTCGTTGTTGAAGTCA-3′). The relative gene expression differences were calculated using threshold cycle (CT) values that were normalized with the GAPDH gene as an internal control in the same sample.

Statistical Analysis

The statistical significance was assessed by permutation t-test (p-value < 0.05) since the distributions of the qPCR do not follow the normal distribution and there are extremely large outliers. Since extremely large outliers distribute differently across three replicated data, we first performed the permutation t-test in each data separately. We also constructed two datasets by taking the median or the trimmed mean of three replicated data. From these five datasets, we selected appropriate GC biomarker candidates by the criteria that comply with a p-value ($p < 0.05$) of the permutation t-test between the qPCR results of GC and NC.

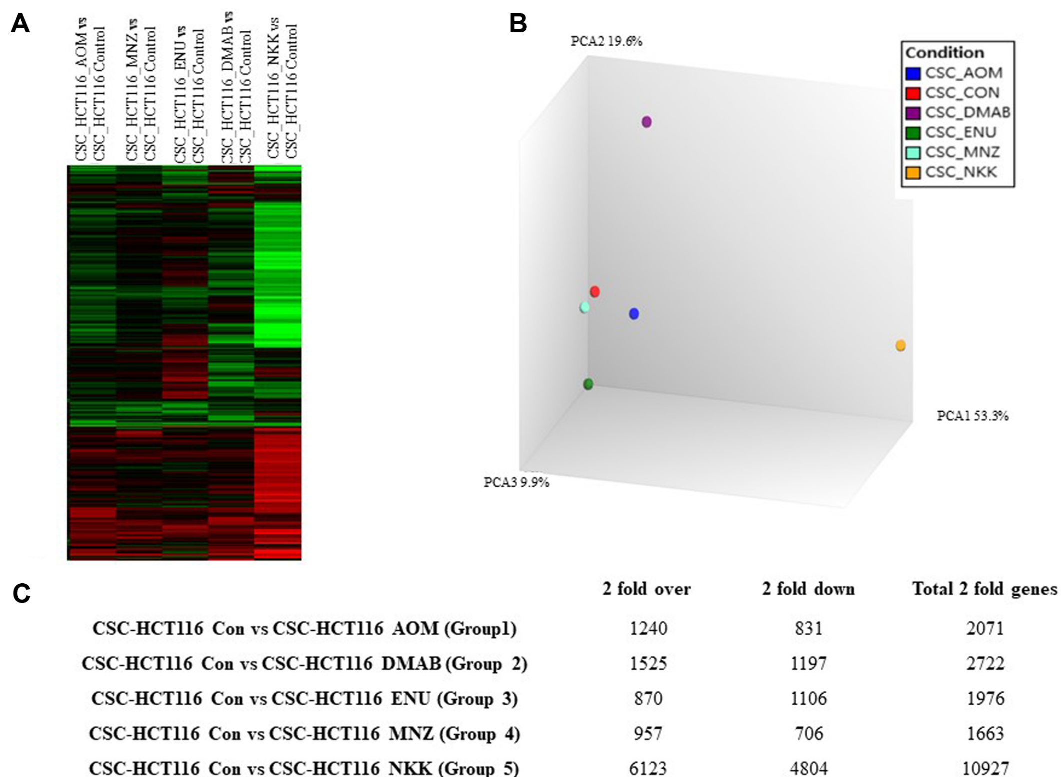
Results

Gene Expression Changes by Compounds in Colon Cancer Stem Cells

The purpose of this study was to identify commonalities in gene expression alterations induced by genotoxic

carcinogens (GCs) with similar modes of action. To determine the optimal concentrations of chemicals including GCs and non-carcinogens (NCs) for colon cancer cells, we selected concentrations based on 1/10 of the concentrations used in mice. The concentrations for mice are reported in the testing data in summary form in the Chemical Carcinogenesis Research Information System, which is searchable on the NLM TOXNET system. We selected the highest dose of chemical for the CCK cell viability assay using the HCT116 colon cancer cell line (data not shown). If the highest dose of chemical exposure did not decrease cell viability by up to 90%, we analyzed the effects of these concentrations (Table 1) on HCT116 colon cancer stem cells. Stem-like cells show non-adherent growth [9]. Similar to previous studies, we used anchorage-independent sphere formation for culturing cancer stem cells [10−12]. It has been suggested that stem anchorage-independent growth promotes cellular carcinogenesis [13] and stem-like cells can be considered as cancer-associated indicators for detecting breast cell carcinogenesis [14]. Based on these reports, we used HCT116 colon cancer stem cells to verify the carcinogenic potential.

Next, we performed microarrays to identify GC biomarker candidates using the GCs AOM, ENU, MNZ, NKK, and DMAB. Gene expression profiles of these compound-treated HCT116 colon cancer stem cells were detected following exposure to each chemical and showed distinct patterns in hierarchical clustering (Fig. 1A). We performed principle component analysis using the five data sets to explore the gene expression variance caused by the chemicals (Fig. 1B). NKK showed the greatest effects, followed by DMAB. Next, the number of significant deregulated probe sets in the five GC groups was determined (Fig. 1C). We selected genes showing similar tendencies in all five GC groups. We considered deregulated gene expression as a change by 2-fold (higher or lower) compared with the vehicle control group (Table 2). After excluding "unknown" probe sets that were not identified by gene symbols and functions, 16 genes were selected. The identified GC biomarker candidates are involved in calcium ion binding, DNA binding, cyclin-dependent kinase activity, nucleotide binding, *etc*.



	2 fold over	2 fold down	Total 2 fold genes
CSC-HCT116 Con vs CSC-HCT116 AOM (Group1)	1240	831	2071
CSC-HCT116 Con vs CSC-HCT116 DMAB (Group 2)	1525	1197	2722
CSC-HCT116 Con vs CSC-HCT116 ENU (Group 3)	870	1106	1976
CSC-HCT116 Con vs CSC-HCT116 MNZ (Group 4)	957	706	1663
CSC-HCT116 Con vs CSC-HCT116 NKK (Group 5)	6123	4804	10927

Fig. 1. Microarray analysis of HCT116 colon cancer stem cells treated with five genotoxic carcinogens (GCs) (azoxymethane (AOM), *N*-ethyl-*n*-nitrosourea (ENU), metronidazole (MNZ), 4-(*n*-methyl-*n*-nitrosamino)-1-(3-pyridyl)-1-butanone (NKK), and 3,2'-dimethyl-4-aminobiphenyl (DMAB)) for 7 days.

(**A**) Hierarchical clustering showing distinct gene expression profiles of the cells. (**B**) Principle component analysis based on the five GCs. (**C**) Two-fold deregulated genes by the five GCs.

Table 2. Two-fold deregulated genes in the microarray.

Gene description	Gene symbol	Gene accession number	GO molecular fuction term	Log ratio [CSC_ HCT116_ AOM vs. CON]	Log ratio [CSC_ HCT116_ DMAB vs. CON]	Log ratio [CSC_ HCT116_ ENU vs. CON]	Log ratio [CSC_ HCT116_ MNZ vs. CON]	Log ratio [CSC_ HCT116_ NKK vs. CON]	Direction of deregulation
S100 calcium-binding protein A7A	S100A7A	ENST00000 329256	Calcium ion binding	-1.4	-1.2	-1.8	-1.3	-1.5	↓
Enolase 1 (alpha)	ENO1	NM_001201 483	DNA binding	-2.1	-2.1	-1.1	-1.7	-2.8	↓
Golgin A7 family, member B	GOLGA7B	NM_001010 917	---	-1.2	-1.1	-1.4	-1.1	-1.3	↓
Pepsinogen 5, group I (pepsinogen A)	PGA5	NM_014224	Aspartic-type endopeptidase activity	1.7	1.8	2.2	1.3	1.3	↑
Secretin	SCT	NM_021920	Hormone activity	1.4	1.2	1.0	1.1	1.2	↑
Membrane-spanning 4-domains, subfamily A, member 4E	MS4A4E	ENST00000 398984	---	-1.6	-1.6	-1.4	-1.0	-1.5	↓
H3 histone, family 3C	H3F3C	NM_001013 699	DNA binding	-1.7	-1.2	-1.1	-2.0	-1.8	↓
Keratin-associated protein 4-6	KRTAP4-6	NM_030976	---	-1.1	-1.1	-1.1	-1.1	-1.2	↓
Zinc finger protein 844	ZNF844	ENST00000 439326	DNA binding	-1.2	-1.4	-1.1	-1.5	-1.2	↓
Cyclin-dependent kinase-like 4	CDKL4	NM_001009 565	Cyclin-dependent protein kinase activity	-1.5	-1.4	-1.2	-1.7	-1.3	↓
RNA-binding protein S1, serine-rich domain	RNPS1	NM_006711	Nucleotide binding	2.0	1.9	1.9	1.9	2.4	↑
Histone cluster 1, H2bh	HIST1H2BH	NM_003524	DNA binding	1.2	1.7	1.2	2.8	1.2	↑
Ribosomal protein S12	RPS12	ENST00000 230050	Structural constituent of ribosome	-1.2	-1.2	-1.4	-1.1	-1.6	↓
Chromosome 6 open reading frame 47	C6orf47	ENST00000 431256	---	2.2	1.4	1.9	2.2	1.4	↑
Carboxypeptidase A4	CPA4	NM_016352	Metallocarboxy-peptidase activity	-1.3	1.1	-1.2	-1.2	-1.4	↓
Phosphatidylinositol-specific phospholipase C, X domain containing 1	PLCXD1	ENST00000 381657	Phospholipase C activity	1.4	1.1	1.1	1.2	1.7	↑

Predictive Gene Selection in Compound-Treated Colon Cancer Stem Cells

After identifying GC biomarker candidates by microarray analysis, we validated these 16 candidates to differentiate GCs and NCs. We performed qPCR analysis using 23 chemicals, including GCs and NCs. To confirm the accuracy of the 16 candidates, we treated HCT116 colon cancer stem cells with 23 chemicals for 7 days and then extracted the mRNA, synthesized the cDNA, and performed qPCR. We selected appropriate GC biomarker candidates with a *p*-value ($p \leq 0.05$) from the permutation *t*-test between the qPCR results of GCs and NCs. Finally, we optimized six genes: *PGA5*, *SCT*, *MS4A4E*, *KRTAP4-6*, *RNPS1*, and *PLCXD1*. We then performed GeneMANIA and GIANT analyses to determine the relationships among the identified candidates. The network consisted of 26 genes, including six identified genes and 20 additional genes identified by GeneMANIA (Fig. 2A). Physical interactions (74.39%), predicted (12.80%), coexpression (6.10%), genetic interaction (3.05%), pathway (1.83%), co-localization (1.22%), and shared protein domains (0.61%) were confirmed by literature searching. Furthermore, GIANT analysis revealed the relationships and predicted pathways of the genes in colon tissue (Fig. 2B). The GC biomarker candidates are involved in RNA processing, RNA splicing, RNA transport, Wnt signaling pathway, *etc.* (Table S1).

Discussion

In this study, we tried to identify specific GC biomarkers

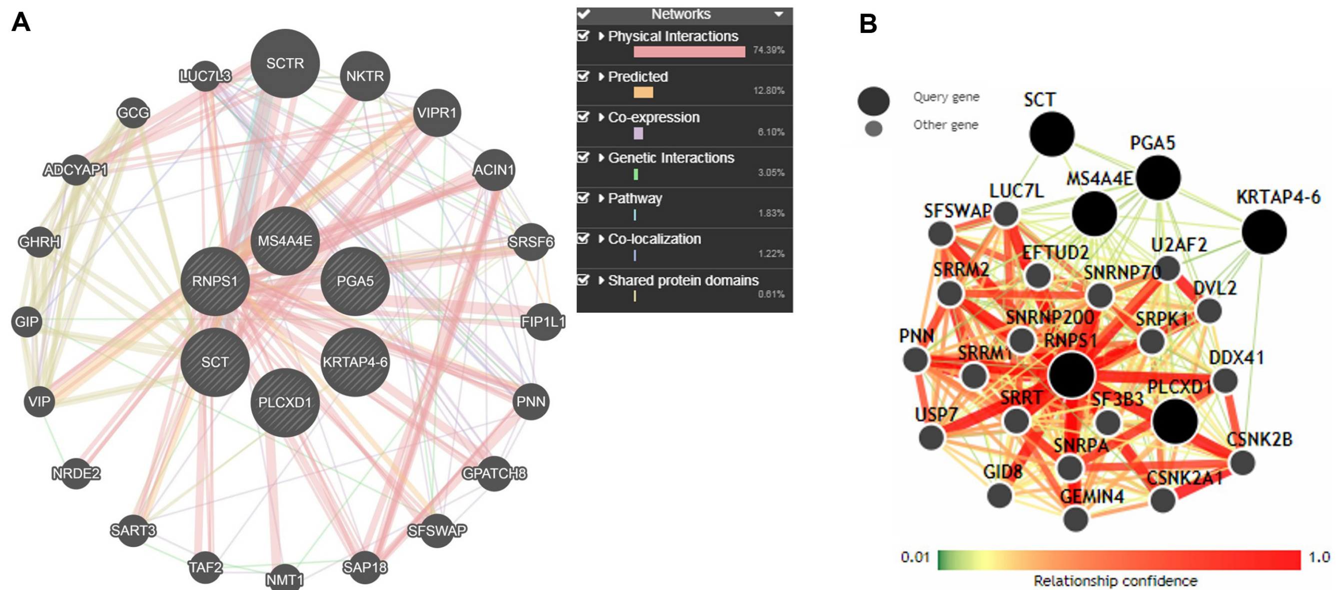


Fig. 2. Network analysis of all interactions showing 2-fold deregulation of genes in microarray analysis.
(**A**) GeneMANIA; Inner circle genes are 2-fold deregulated genes and outer circle genes are related genes. (**B**) Functional network built using GIANT in colon tissue (Genome-scale Integrated Analysis of gene Networks in Tissues).

using in vitro genotoxicity tests. The carcinogenicity of chemicals is an important life-threatening hazard to humans. Conventional carcinogenicity tests include long-term and short-term rodent assays and in vitro cell-based assays and often show ambiguous results in genotoxicity tests [15]. Therefore, alternative carcinogenicity screening assays are needed. Various alternative tests including microarray and other toxicogenomics approaches have been developed. However, these arrays require specialized equipment and highly skilled bioinformatics approaches, limiting the evaluation of carcinogenicity test results. In this study, we evaluated GC biomarkers by qPCR, which is a cost-effective approach. Cumulative exposure of human and mouse cells to carcinogens leads to the progression of cellular carcinogenesis and causes distinct genetic changes [14, 16−18]. Therefore, cancer-associated properties can be considered as measurable targeted endpoints for studying cellular carcinogenesis progression. Non-adherent cancer cells can be developed into stem-like cells and show anchorage-independent growth, as reported previously [14]. Stem-like cells have been shown to play major roles in cancer development [2]. Thus, cancer stem-like cells can be used as a tool for evaluating carcinogenicity. Our results suggest that colon cancer stem cells and their GC biomarkers can be used as carcinogenesis indicators for detecting chemical carcinogenicity towards colon cancer cells. We evaluated the mechanisms functioning in carcinogen-treated colon cancer stem cells and developed an alternative carcinogenicity test. Using qPCR analysis, we selected six predictive genes (*PGA5, SCT, MS4A4E, KRTAP4-6, RNPS1*, and *PLCXD1*) from among the 16 genes predicted by microarray analysis based on data following treatment with 17 GCs and 5 NCs. Using DiseaseConnect (http://disease-connect.org/), we explored the connectivity of these selected genes on cancer. This disease connectivity network is based on Genome-Wide Association Studies (GWAS) data, clinical records, OMIM records, and metabolic networks [19]. Some of the identified GC biomarker candidates, *SCT, RNPS1*, and *PLCXD1*, have connectivity on diverse cancers. A previous study reported that *SCT* injection can induce gastrin release, which is a common finding in gastrinoma [20], *RNPS1* has a function on the composition and stability of numerous cellular mRNA, that can modulate tumor growth and progression [21] and *PLCXD1* regulates melanoma cell growth [22]. In this study, those genes were upregulated in colon cancer cells by five GCs. In this regard, the identified candidates can be used as GC biomarkers. They clearly differentiated GCs and NCs in distinct cellular signaling pathways. This study presents a unique approach for determining colon cancer carcinogenesis using new GC biomarkers. These measurable biomarkers can be used as new endpoints for detecting carcinogenesis. Our alternative carcinogenicity screening assay using qPCR and colon cancer stem cells will be useful

as an additional carcinogenicity testing strategy. This assay may improve in vitro carcinogenicity testing. In the future, the biomarker candidates can be used to differentiate GCs from NCs. Further studies should be performed to compare the present NC- and GC-specific biomarker candidates.

Acknowledgments

This research was supported by grants (16182MFDS391) from the Korean Ministry of Food and Drug Safety (2017) and the basic program (2018R1A2B2001225) of the National Research Foundation of Korea (NRF).

Conflict of Interest

The authors have no financial conflicts of interest to declare.

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