Predicting Gene Interactions in Tumor Microenvironment using Patient Derived Spheroid Primary Cell Culture

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Research note

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Abstract

Objective

Cancer is a heterogenic disease from genetic to individual levels, which induced a variety of therapheutical research and disease progression. Cancer heterogeneity were also observed in most of solid tumors including colorectal cancer. Tumor growth depends on intra-tumoral complexity in which tumor cells surrounded by several cells in their nische known as tumor microenvironment. In this study, we are using spheroid derived primary culture derived from patients to predict expression pattern of tumor growth in their microenvironment in vitro. The gene list were data which obtained from our other “on going publications”

Results

In this study, we cultured patient-derived primary cells for 7 days and analyzed 24 genes that positively expressed in this in vitro system. Using STRING, we presented interactions between proteins which later may predict the interaction between the cells. The result shown that the cells actively using carbon metabolism to maintain energy, to produce several protection mechanism against oxidative stress to evade apoptosis, and to stimulate several protein related to epithelial to mesenchymal transition and angiogenesis in vitro.

Introduction

Colorectal cancer (CRC) is a malignancy in large intestine tissue, consisting of the colon and/or rectum. (1) Colorectal cancer is the third leading cause of all new cancer cases and is the leading cause of cancer-related death worldwide.(2) Apart from the high mortality rate, CRC is also causing problems due to the incidence of relapse and drug resistance.(3, 4) Tumor growth may be influenced by multi factorial factors such as mutations inside the tumor, expression pattern and also influenced by its environment.(5, 6, 7) The tumors were interacting with other cells inside the nische including tumor associated fibroblast, tumor associated immunity or Tumor Associated Macrophages (TAM), tumor associated netrophils, endothelial cells and T cells which together contributed to tumor progressions.(8–17) This complex mechanism also influenced tumor sensitivity towards distinct treatments such as radiotheraphy and chemotherapheutical agents.(18, 19)

Tumor microenvironment can be studied using biopsies samples to understanding celullar compositions as well as protein expression inside cell nische. (20) Such strategies were advanced for the prediction of patient survival, progression or respond to certain therapheutical options in clinical settings.(21, 22, 23) However, biopsy does not virtually describe how the cells grow. One of the most potential tools to predict microenvironment is spheroid cultures, since the spheroid culture has more resemblance to physiological condition (24). It also supports mesenchymal stem cell in vitro, and enable to study tumor angiogenesis in vitro. (25, 26, 27) Furthermore, 3D and spheroid cultures allow cross talk of cancer cells to endothelial

cell and allow the study of macrophage plasticity. (28, 29) Therefore, our main idea was to use spheroid based primary culture derived from biopsies in order to find out what type of genes that expressed in order to predict tumor budding behaviour or growth pattern in vitro.

Methods

1. Patients and Sample Collections.

Samples were collected from patients who gave their written informed consent for inclusion before participate in the study. The study was conducted in accordance with the Declaration of Helsinki, and the protocol has been approved by the Ethical Committee of Faculty of Medicine, Universitas Indonesia. Protocol ID : 20-04-0643

2. Tissue Extractions

The biopsies were extracted using mechanical digestions by dicing the tissue into 3-5 mm^3. Tissue then dipped into 0.1 % of Povidine Iodines in Dulbeco's Modified Eagle Medium (DMEM, Gibco, USA). The fragments were then extracted with 10 cc syringe plug and centrifuged briefly at 300 x g for 5 minutes in RT. The pellet were suspended with complete DMEM (Gibco, USA) supplemented with 10 % of Fetal Bovine Serum (Gibco, USA) and 1 % of Antibiotic-Antimycotic solutions (Gibco, USA).

3. Spheroid Culture

The cell suspensions were counted using trypan blue exculsion assay and 10,000 of viable cells were seeded in 96 well u-plate (Nunc-Sphera, Thermoscientific, USA). The cells were cultured in complete medium for approximately 7-9 days without changing the medium. The morphology were captured using inverted microscope of Axio Carl Zeiss using Zen blue 2.1.edition software in 5x maginifications.

4. RNA Extractions and cDNA synthesis.

The cells were harvested by pippeting and suspended by centrifuging at 300 x g for 5 minutes. The RNA were extracted using RNA Miniprep plus (Zymo Research, USA) according to manufacturer's protocol. The RNA were directly reverse transcribed using RT First Strand Kit (Qiagen, Hildenburg, Germany) according to the standard protocol.

5. RT-Profiler using Cancer Pathway Finder.

The quantitative expression were performed using Human Cancer Pathway Finder (Qiagen, Hildenburg, Germany) using Sybr Green (Qiagen, Hildenburg, Germany) according to manufacturer's protocol. The PCR were done in ABI 7500 FAST and data were collected according to recommendations set up. The positivity were confirmed while amplifications reached above background signal and detected between cycle 20-39.

6. Protein Networking Analysis.

The list of positively expressed were then analyzed using STRING and its predicted pathway were obtained.

Results

1. Establishment of Primary Spheroid culture.

Primary cultures were able to be generated at day 7. In this method, we were able to generate spheroids. Figure 1 shows heterogeneity of primary culture under in vitro conditions (10,000 cells/well). Complexity of the primary cells is shown by the morphology of epithelial cells which is the rounded cells. In our experiments, using smaller density such as 1,000 cells per well generated a more compact spheroid as shown in figure 1.

2. Detection of Positive genes using RT-PCR.

The genes positively expressed were detected using PCR at cycle 20-40. The list of genes and its CT were shown in figure 2 while most of the genes were expressed between CT 35 and 40.

3. Protein Networking Analysis.

The gene list were analyzed using STRING and several interactions were found as shown in figure 3. Two genes with no interactions were ACSL4 and CCND3.

Discussion

The protein network shown in figure 3 indicates at least several pathways have correlations. We first describes the metabolism in cancer. Gene expressions playing role in metabolisms were G6PD, PFKL and LDHA which mostly found in carbon metabolism.(31) Other proteins were HRPT1, while protein ACSL4 were not connected to any networking. PFKL known as phosphofructokinase is a rate limiting enzyme in glycolisis which supports glucose metabolism. It has strong connection with LDHA protein in anaerobic metabolism known as warburg effect to maintain tumor proliferations.(32,33) Warburg effect produces energy for the cells to maintain the cell growth and and its fast proprety makes it commonly used by tumor cells.(34) This effect also related with hypoxia pathway, which may occured inside the core of spheroid that mimics tumor property.(35,36)

Beside its role in glucose metabolism, PFKL and G6PD expressions revealed deeper knowledge on cancer metabolism. G6PD or Glucose-6-phosphate Dehydrogenase is a rate limiting enzymes of pentose phosphate pathway. It is a pathway that supports ribose-5-phosphate synthesis required to sintesize nucleotide.(31) Another important finding is the connection with HPRT1, a gene responsible in *de salvage* pathway to provide nucleotides.(37) Therefore, the tumor may activate pathway that produce 5-ribose phosphate for nucleotide backbone and actively reproduce *de salvage* by expressing HRPT1. The

importance of G6PD is its role to provide NADPH for power reducer. It is neccesary to regenerate fatty acid. Inside the networking there were no connections or any other informations related to fatty acid synthesis. However, ACSL4 which is known to ligate long fatty acid to coenzyme A and to synthesize long fatty acid, is essential in metabolism, modification of membranes structures, and proliferation.(38) Together with G6PD expression, we assumed that NADPH was also used by cells for the synthesis of fatty acid.

Interestingly, SOD1 known as cytosolic superoxides were also expressed. this may indicate that the tumors protect its cells from reactive superoxide generated as metabolism byproduct by expressing the SOD1. The SOD1 has a protective property against anion superoxide produced by several metabolic pathway.(39) Taken together with G6PD expression, the tumor cells may provide higher reducing power such as NADPH protein expression and high ROS productions inside the cells to support the tumor growth.

The network analysis conveyed positive expression of CASP7, CFLAR and XIAP. CASP7 overexpression were known to induced apoptosis. (40,41) However, in cancer cells overexpression of CASP7 usually indicates poor prognostic factos. Other protein expressed was CLFAR, known as programmed cell death. It modulates apoptotic pathway by regulating caspase-8 activations. (42) Lastly, XIAP protein, also knowns as modulator. This protein regulates apoptosis by directly bind to binding pocket of caspase-7 and caspase-3.(43,44) The proteins were known to inhibits proteasomal degradations that promotes autophagy(45).

To support cell growth, the cells are mostly maintaining complex mechanisms. One of the main mechanism is angiogenesis. TEK, ANGPT1, VEGFC and FLT1 conveyed that the cells support angiogenesis related pathways. TEK, also known as Tie2, were expressed on tumors vasculature. While ANGPT1 overexpression is related to the increasing tumorigenicy of breast cancer cell lines. (46) TEK and ANGPT1 expression may indicate that spheroids may describe tumors and endothelial crosstalk communications. VEGFC overexpression is known to correlate with poorer survival as it supports tumor growth.(47) Moreover, FLT1 is known to support not only tumor growth but also metastasis and mostly due to its interactions with macrophages.(48) Interestingly, SERPINF1 expression, known as anti-angiogenic factors, may be released by certain cells inside this spheroid cultures to suppress the tumor growth.(49) This is interesting, since suppresion of this SERPINF1 could induce tumor promoting phenotype of cancer associated fibroblast.(50)

We also found interactions of FOXC2 and CDH2. FOXC2 may be related to TEK and angiogenesis since FOXC2 supports angiogenesis and disease progression (51). Its connection with CDH2 may be related to reprogramming event of epithelial to mesenchymal transitions (EMT), indicating celullar plasticity.(52) CDH2 were related to aggresive phenotypes and protections of microenvironment against therapheutical agent.(53) Moreover, the expression of SNAI3 and KRT14 related to CDH2, may indicate EMT process inside the spheroids.(54) Despite its interactions were closely related to apoptosis, PINX-1 and TINF2

expression may be related to tumor suppresion effect inside the spheroids.(55) Therefore, at certain extend some cells may have tried to suppress the tumor growth.

However, the expression of IGFB3 and CCND3 were found. IGFB3 may be related to hypoxia related signaling, induced by hypoxic microenvironment.(56) Serum IGFBP3 is known to correlate with VEGFC and may be related to metastasis insidence.(57) IGFB3 were also shown to bind with several growth factors.(58) Lastly, CCND3 expression may be related to malignancy and tumor growth capacity, since its known as poor overall survival prognostic.(58)

Limitations

- The spheroid culture were able to be conducted, through using one patients as representative data. To gain more information, at least two or three patients should be analyzed using the same assay.
- Genes interactions may not representate the true metabolism, therefore metabolited should be at least measured in the future.
- The multiple cell staining were not conducted, therefore genes and cell interactions were only limited to genes interactions.

Abbreviations

ACSL4	Acyl-CoA Synthetase Long Chain Family Member 4
ANGPTI1	Angiopoietin 1
CASP7	Caspase-7
CCND3	Cyclin D3
CDH2	Cadherin 2
CFLAR	CASP8 And FADD Like Apoptosis Regulator
CRC	Colorectal Cancer
CT	Cycle Threshold
DMEM	Dulbecco's Modified Eagle Medium
EMT	Epithelial-Mesenchymal Transition
FLT1	Fms-related tyrosine kinase 1
FOXC2	Forkhead Box C2
G6PD	Glucose-6-Phosphate Dehydrogenase
HRPT1	Hypoxanthine Phosphoribosyltransferase 1
IGFB3	Insulin-like growth factor-binding protein 3
KRT14	Keratin-14
LDHA	Lactate Dehydrogenase A
PFKL	phosphofructokinase, liver type
PINX-1	IN2/TERF1-interacting telomerase inhibitor 1
RT-PCR	reverse-transcriptase polymerase chain reaction
SERPINF1	Serpin Family F Member 1
SNAI3	Snail Family Transcriptional Repressor 3
SOD1	Superoxide Dismutase 1
TAM	Tumor Associated Macrophage
TEK	TEK Receptor Tyrosine Kinase
TINF2	TERF1 Interacting Nuclear Factor 2
VEGFC	Vascular Endothelial Growth Factor C
XIAP	X-linked inhibitor of apoptosis protein

Declarations

Ethics Approval and Consent to Participate

This is a study involving human tissue with ethical approval granted by Ethical committee from University of Indonesia, Protocol ID : 20-04-0643.

Availability of the Data Sets and Material.

The datasets of gene lists and figure already listed within this manuscript. STRING analysis of those list can be analyzed using the online tools.

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Consent For Publication.

Non Applicable.

Competing Interest.

The authors declare that they have no competing interest.

Author’s Contribution

MA, APU and SAN concept and design the work. SAN and DRN acquire and analyse the data. MA and DRN interpret the data. All authors have read and approved the final manuscript.

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Figures

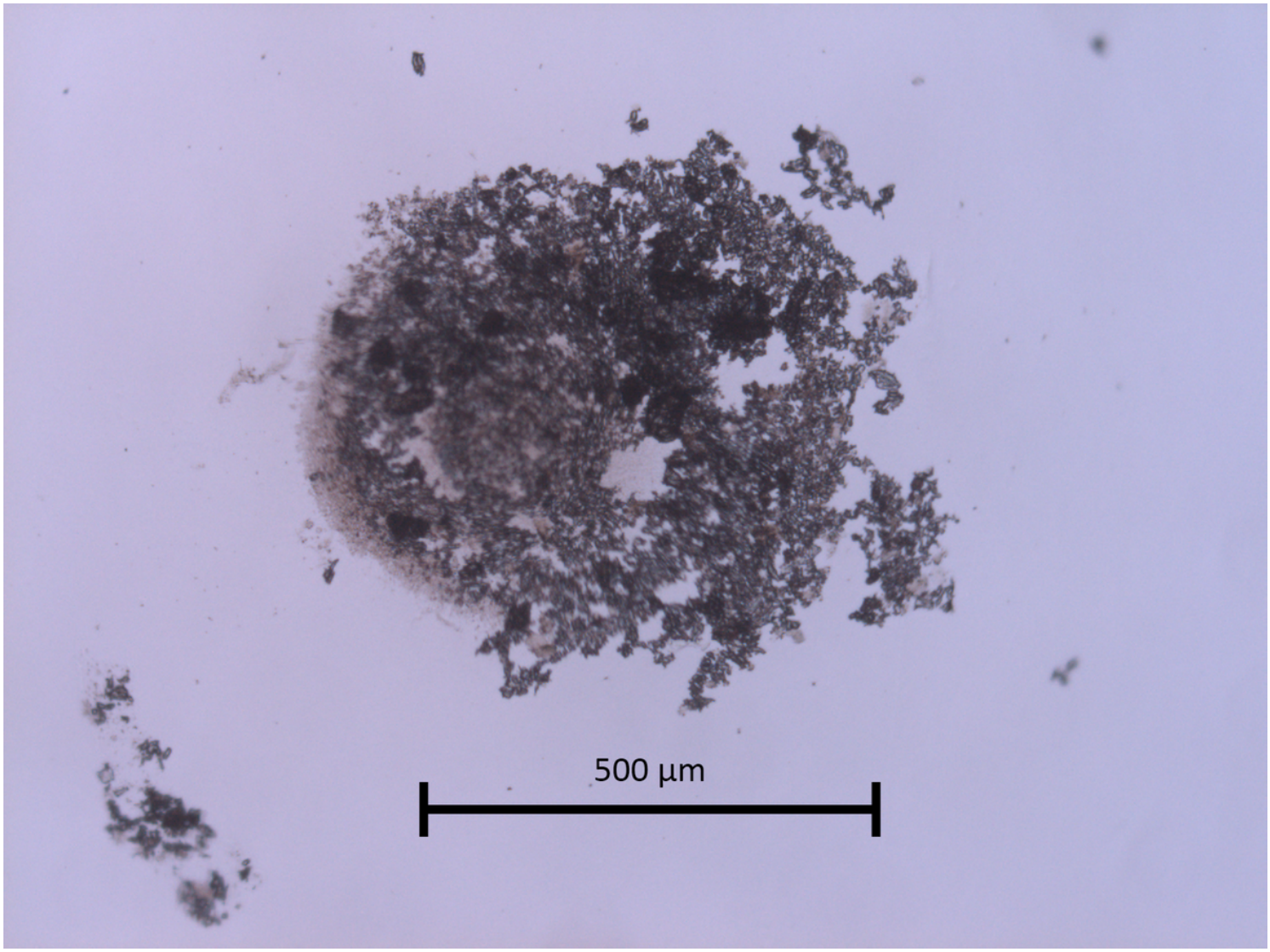

Figure 1

Spheroid at day 7 of culture with 1,000 cells per well.

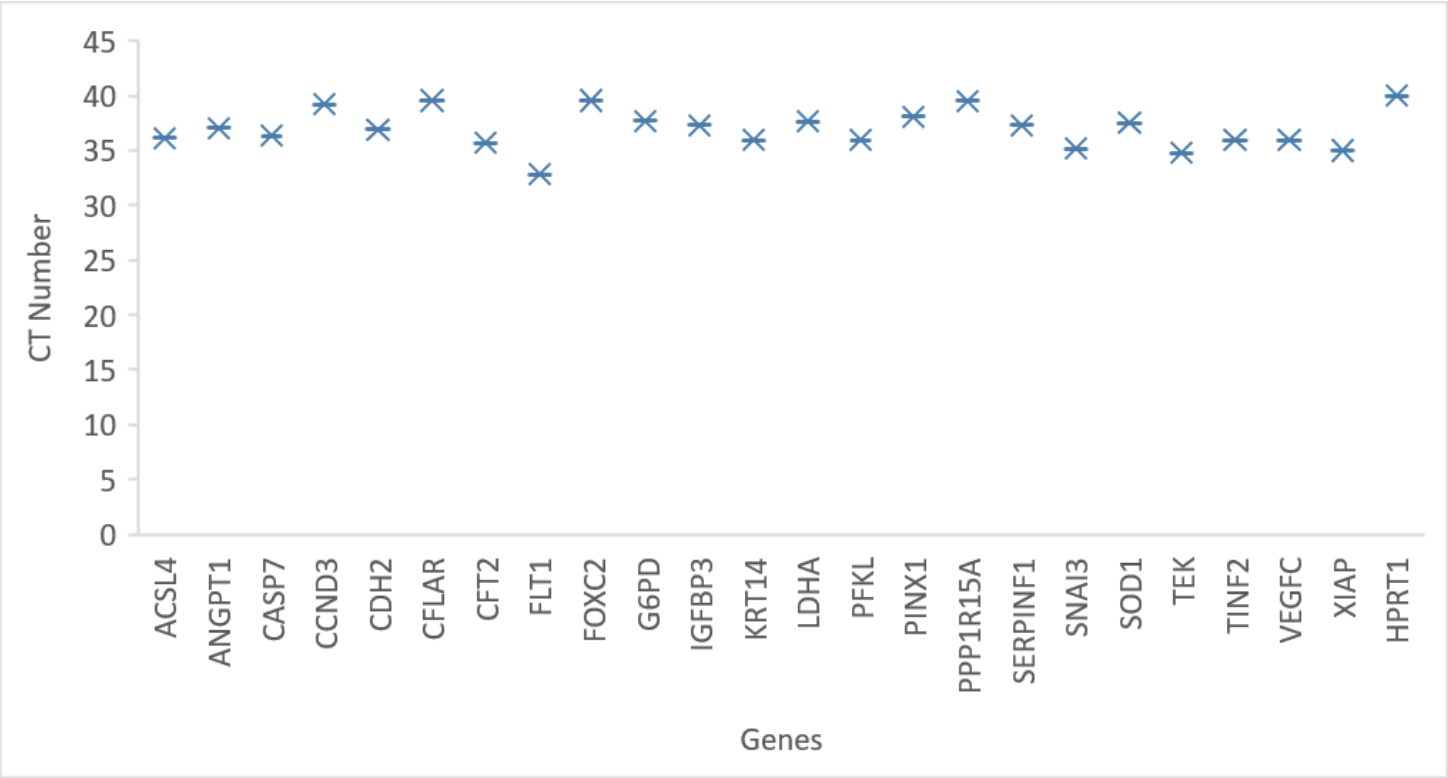

Figure 2

Genes expression detected in Human Cancer Pathway Finder. The results showed 24 positive genes in this spheroid culture.

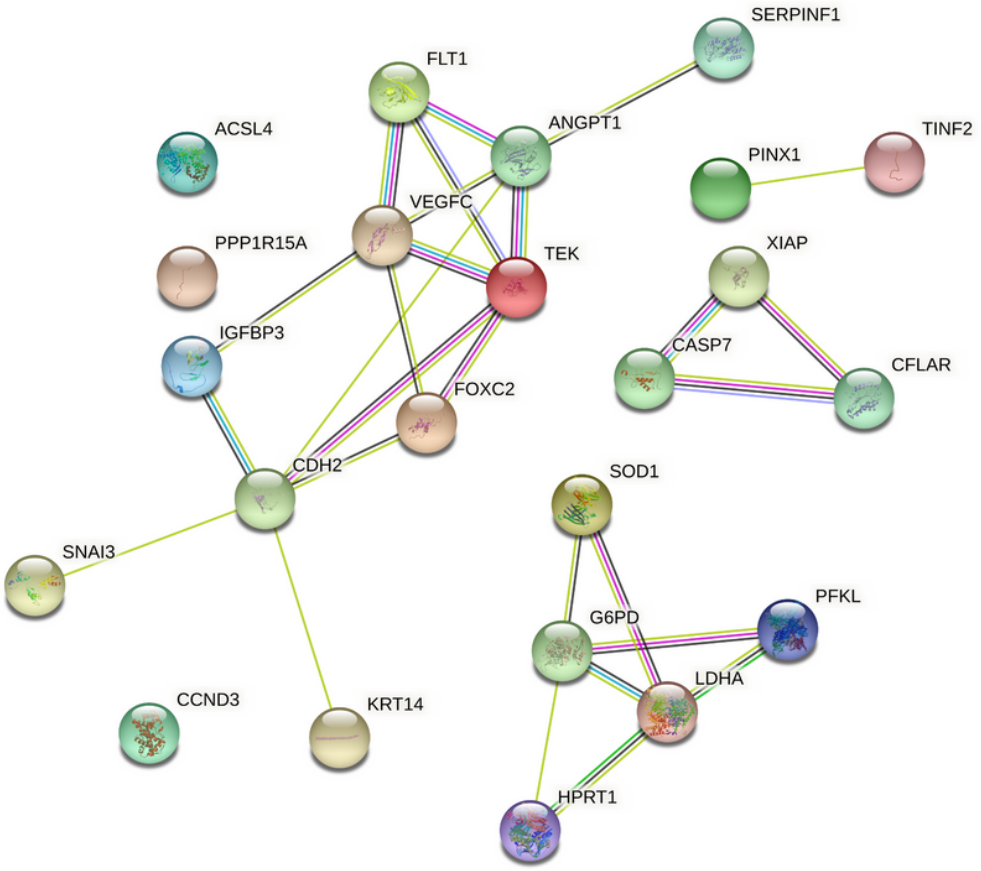

Figure 3

Protein interaction prediction of 24 expressed genes using STRING. The networking conveys proteins playing role in common metabolism, apoptosis, cancer related metabolism.(30)

Supplementary Files

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