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Transcriptional Analysis-Based Integrative Genomics Approach to Identify Tumor-Promoting Metabolic Genes

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Abstract

Metabolic regulation can play key role in normal and pathological states. In particular in cancer cells, alterations in metabolic pathways can drive the growth and survival of cancer cells. Among these alterations, many occur at the transcriptional level leading to the overexpression of metabolic genes. However, not every metabolically upregulated genes may be necessary for tumor growth. Therefore, functional validation approaches are required to distinguish metabolically overexpressed genes that are necessary for tumor growth versus the ones that are not. One of the experimental approaches to do this is to use the approach of RNA interference to systematically survey the transcriptionally upregulated metabolic genes for their requirement in tumor growth. Here, we describe an integrative genomics approach to identify metabolic genes that are necessary for tumor growth. The approach we describe is a general integrative genomics approach that combines bioinformatics-based identification of overexpressed metabolic genes in cancer patient samples and then uses RNAi-based knockdown approach to identify genes that are necessary for tumor growth.

Keywords

RNA interference; Metabolism alteration; Gene expression analysis

1 Introduction

It is now well appreciated that cancer cells arise due to not small set of changes in normal cells, rather a series of multiple changes that make cancer cells adaptable to grow and expand in vivo and counteract a variety of stress and inhibitory situations. Collectively, these features are recognized as hallmarks of cancer. From the time of Otto Warburg discovery of aerobic glycolysis in cancer cells, it became clear that cancer cells show alterations in metabolic pathways and these changes seemed more than just passenger alterations [1]. Since the discovery of “Warburg effect,” several studies have identified, and implication metabolic alteration in almost all metabolic pathways in cancer cells and their role has been shown in almost all aspects of cancer growth and metastasis as well as therapy response [1]. In most cases candidate-based approaches have been used to identify genes and pathways that are important for metabolic regulation of tumor growth. However, in most cases these targeted approaches do not allow a large-scale survey of metabolic alterations in cancer

cells. Therefore, systematic approaches to identify metabolic alterations necessary for tumor growth are necessary.

Availability of a large number of microarray and RNA-sequencing data from the patient-derived tumor samples and normal control in public domain gives an ample opportunity to identify metabolic alterations in cancer cells. Once metabolic genes that are transcriptionally upregulated in patient-derived cancer cells are identified, this information can be combined with a targeted RNA interference screening approach to identify which of the transcriptionally upregulated genes that are necessary for tumor growth.

Several factors must be considered when analyzing the microarray or RNA-sequencing datasets for patient-derived cancer samples and designing a successful RNAi screening. General guidelines for choosing the appropriate RNAi library (e.g., shRNA vs. siRNA, 20 retroviral vs. lentiviral) and screening strategy (single well vs. 21 pooled format, positive vs. negative selection, etc.) have been covered in detail elsewhere, and the reader is referred to several excellent reviews on these topics [2–5].

When planning a targeted RNAi screening for transcriptionally overexpressed metabolic genes, there are several critical parameters to consider. First, it is really important to choose the right cancer datasets. Preferably, multiple datasets should be analyzed for each cancer type, and genes that are commonly overexpressed should be considered for analysis. Second, at least two and ideally three sequence-independent short-hairpin (shRNA) or small interfering (siRNA) should be used to confirm the observed effect. Third, it is important to choose a cancer cell line in which the gene of interest is overexpressed, similar to that observed in the case of patient samples. Fourth, it is fundamental to choose an appropriate negative control to evaluate the background of the screen. In most cases, a control non-silencing shRNA or luciferase siRNA is suitable. Finally, the choice is also very important, as these will allow the researcher to clearly distinguish between false and true positive.

Here, we describe an integrative genomics approach to identify metabolic drivers of tumor growth. The approach described here is a general screening strategy that could be used to study and identify metabolic driver of tumor growth.

2 Materials

2.1 Analysis of Patient- Derived Cancer Sample Datasets

1. Gene Expression Omnibus (<http://www.ncbi.nlm.nih.gov/gds>) (publically available).
2. ArrayExpress (<https://www.ebi.ac.uk/arrayexpress/>) (publically available).
3. Oncomine (<https://www.oncomine.org/resource/login.html>) (license required for detailed dataset analysis).
4. The Cancer Genome Atlas (<http://cancergenome.nih.gov/>) (request to access is needed).
5. International Cancer Genome Atlas (<https://icgc.org/>).

6. cBioPortal (<http://www.cbioportal.org/>).

2.2 Generation of Retroviral Particles, Determination of the Multiplicity of Infection, and Cell Infection

1. Gene-specific shRNAs (preferably TRC library shRNAs cloned in pLKO.1 vector) and a control shRNA, such as a nonspecific shRNA.
2. 293 T cells (American Type Culture Collection).
3. pMD2.G plasmid (Addgene).
4. psPAX2 plasmid (Addgene).
5. Transfection reagent, such as Effectene (Qiagen).
6. 0.45 mM filters.
7. Culture medium: DMEM high glucose (1×, liquid, with L - glutamine and sodium pyruvate), 10% FBS, and penicillin–streptomycin.
8. Puromycin.
9. Cancer cell line of choice, for example, A735 for melanoma-related experiments.

2.3 Isolation of Total RNA- and shRNA-Specific Gene Knockdown Validation by RT-qPCR

1. Trizol reagent (Invitrogen).
2. RNeasy mini columns (Qiagen).
3. Gene-specific primers.
4. ProtoScript First Strand cDNA synthesis kit (New England Biolabs).
5. Power SYBR green mix (Invitrogen).
6. 384-well qPCR plates.
7. Clear qPCR plate sealing film.
8. Quantitative PCR machine.

2.4 Soft-Agar Assay to Measure Tumorigenic Potential of Cancer Cells Expressing Specific shRNAs

1. DMEM high-glucose media with L-glutamine powder for 1 L (Sigma-Aldrich).
2. 100× penicillin and streptomycin.
3. Trypsin-EDTA (0.25%) (Invitrogen).
4. 1× phosphate-buffered saline.
5. Low gelling agarose.
6. Sterile 6-well tissue culture plates.
7. CO₂ incubator.

8. Water bath.
9. Staining solution (0.05% crystal violet, 40% methanol, 10% acetic acid, and 50% double distilled water).
10. De-staining solution (40% methanol, 10% acetic acid, and 50% double distilled water).
11. Inverted microscope.

2.5 Athymic Nude Mice-Based Tumorigenesis Experiments to Determine the Role of Tumor- Promoting Genes Validated from Soft-Agar Assay In Vivo

1. Athymic nude mice NCR sp/sp (Taconics).
2. Insulin syringe with the 23-gauge needle.
3. Alcohol wipe.
4. Clear air BSL2+ level animal facility.
5. BSL2 laminar airflow.

3 Methods

3.1 Cancer Dataset Analysis

Identify the dataset for analysis using one or more of the databases listed in the Subheading 2.1. GEO datasets available at <http://www.ncbi.nlm.nih.gov/gds> and can be freely downloaded. Most datasets available through GEO can be analyzed using GEO2R. A detailed instruction for using GEO2R is provided at <http://www.ncbi.nlm.nih.gov/geo/info/geo2r.html>, and a video for using GEO2R is also available through <https://www.youtube.com/watch?v=EUPmGWS8ik0>. (see **Note** ¹).

3.2 Generation of Lentiviral Particles

1. Plate 100,000 293 T cells in 12-well tissue culture dishes using DMEM high-glucose media containing 10 % fetal bovine serum and penicillin and streptomycin. One well each can be used for gene-specific shRNAs and for non-silencing shRNA.
2. After 36 h of plating, transfect cells with 0.5 µg of shRNA plasmid DNA, 0.5 µg of psPAX2, and 0.25 µg of pMD2.G using Effectene transfection reagent as per the suppliers' information.
3. After 48 h of transfection, collect the culture supernatants, which contain lentiviral particles.
4. Filter the culture supernatants using 0.45 µm filters. Aliquot 0.5 ml supernatant into 1.5 ml microfuge tubes and freeze at –80 °C (see **Note** ²).

¹It is recommended that multiple gene expression datasets are analyzed for each cancer types and this allows data and patient sample collection biases. Also, it is recommended to focus on genes that are upregulated in multiple gene expression datasets compared to the ones that are present only in one.

3.3 Infection and Selection of Cells After Transduction with Lentiviral shRNA Particles

1. Plate 100,000 cancer cells of choice in 12-well plates in 1 ml of culture media recommended for growing the given cancer cells. If you are testing five gene-specific shRNAs, then the total number of wells required for the experiment will be six, which includes one well for the control-nonspecific shRNA.
2. After 24 h, transduce the cells with 100 μ l of supernatant containing lentiviral shRNA particles (*see Note*³). For this purpose, add the 100 μ l of lentiviral shRNA particles containing supernatant directly to the media.
3. After 24 h of injection, change the media and add fresh culture media with 10 % FBS and penicillin and streptomycin.
4. After 24 h of changing the media, add puromycin (range 0.2 μ g/ml to 2.0 2 μ g/ml) to enrich for cells that carry integrated shRNAs (*see Note*⁴). Change the media every 3 days with fresh puromycin. For most cells puromycin selection is complete by 7–10 days. Use uninfected cells as controls to ensure that the selection is complete.

3.4 Validation of Knockdown Efficiency for shRNAs

1. Isolate total RNA from cells expressing individual gene-specific shRNAs as well as nonspecific shRNA using Trizol reagent as per the manufacturers' instructions.
2. Purify the total RNA using the Qiagen RNeasy mini column as per the manufacturers' instructions.
3. Perform cDNA synthesis using ProtoScript First Strand cDNA synthesis kit as per the manufacturers' instructions. Dilute the cDNA to two times after the reaction is over. For example, a 20 μ l reaction should be diluted to 40 μ l.
4. To determine the knockdown efficiency of shRNAs, perform the quantitative PCR. A typical reaction in a 384-well qPCR plate will be as following:

Components	Volume
2 $\times$ Power SYBR green mix	5 μ l
cDNA	2 μ l
Gene-specific primer mix	1 μ l
Double distilled H ₂ O	2 μ l

²It is important to note that freeze-thawing will lead to drop in virus titer. Therefore, we recommend avoiding multiple freeze-thaw cycles. Storing viral supernatant in the form of aliquots at -80°C freezer prevents the drop of viral titer and improves the infection.

³Depending upon the titer of lentiviral supernatant, a smaller or larger amount of supernatant can be used.

⁴It is recommended that a kill curve is performed using different concentrations of puromycin ranging from 0.1 to 2.0 microgram/ml puromycin to determine the right puromycin concentrations for a given cancer cell line for the selection.

5. The relative fold changes in mRNA expression can be calculated using the comparative C_T method [6]. The amount of target gene expression under each indicated condition should be normalized to the expression of human or mouse *GAPDH* as necessary. Relative gene expression among treatment conditions should be calculated using the formula: -2^{-C_T} . PCR efficiency of target genes was matched to the PCR efficiency of *GAPDH* by ensuring that log input versus C_T had a slope of zero.

3.5 Soft-Agar Assay to Determine the Tumorigenic Potential of Specific shRNA-Expressing Cancer Cells

1. Select the cells with shRNAs that shows strong knockdown efficiency. Preferentially shRNAs having 50% or more gene knockdowns should be used. Also, include nonspecific shRNA-expressing cells. Use cells that are from 50 to 70% confluency for soft-agar assay.
2. As a first step generate the base agar layer of 0.8 % agar. To do this mix equal volume of 1.6 % low gelling agar in water, and mix it with 2× DMEM media containing 2× 10 % FBS and 2× penicillin–streptomycin. Make sure to mix the 1.6% agarose at no higher than 45 °C to avoid degrading the heat-labile component in culture media. Pour 2 ml/well of the agarose and culture media mix to the 6-well plate.
3. Allow the base agar layer to solidify. Typically it will take about 1 h for base agar layer to solidify.
4. Once the base agar layer solidified, plate the cells (typically in the range of 2500–10,000 cells/well depending upon the cell lines) in 0.4% agarose. To do so, mix 750 µl of 0.8% low gelling agarose with 750 µl of 1× DMEM with 10% FBS and 1× penicillin–streptomycin containing desired number of cells.
5. Allow the top agar to solidify for 30 min and then transfer the plates to the CO₂ incubators.
6. After 24 h of plating the cells, add 1 ml of cell culture media and continue to feed the cells with 1 ml media every 3 days. Typically, it takes 2–3 weeks to obtain right size colonies in soft- agar assay.
7. At the end of experiments, stain the soft-agar colonies with staining solution (0.05% crystal violet solution in 40% methanol, 10% acetic acid, and 50% water) for 2 h. Remove the staining solution and de-stain the plate using de-staining solution (40% methanol, 10% acetic acid, and 50% water) until the colonies start to become visible.
8. Scan and count the number of colonies and also acquire images at 4× and 10× resolution using inverted microscope. The less number of colonies or smaller number of colonies in a soft-agar assay upon the knockdown of a given gene is reflective of the role of a gene in facilitating tumor growth. A secondary assay, potentially an in vivo tumorigenesis assay should be used to confirm this role. Below, we describe how to perform a typical in vivo tumorigenesis assay.

3.6 Athymic Nude Mice-Based Tumorigenesis Experiments to Determine the Role of Tumor- Promoting Genes Validated from Soft-Agar Assay In Vivo

As a secondary validation for the soft-agar results, we recommend to perform the human cancer cell line xenograft-based in vivo tumorigenesis assay using immunocompromised mice. This can be achieved as below:

1. Obtain athymic nude mice from a commercial supplier approved by your institute to avoid long delays in quarantine. For examples, most places direct supplies from Taconic or Jackson Laboratories. At least five mice per group should be used for obtaining statistically significant result. For a more accurate estimate for the animal number, a power analysis can be performed with the potential effect size included in the analysis. A typical power analysis can be performed using the following website: <http://powerandsamplesize.com/>.
2. Grow the cells in required amount. Typically five to ten million cells are injected per mice subcutaneously (*see Note*⁵).
3. Label the mice with ear tags for easy identification.
4. Wipe the flank of athymic nude mice with ethanol wipes, and then using insulin syringe with attached needles, inject five to ten million cells expressing gene-specific shRNAs subcutaneously into the flank of athymic nude mice. As a control, a same number of cells expressing control shRNAs need to be injected in mice, and tumor growth should be compared with the gene-specific knockdown shRNAs.
5. Allow the subcutaneously inject cells to form a tumor and monitor the tumor growth. Typically tumor growth is monitored for the period of 2–3 months depending upon the growth rate of tumor cells.
6. Measure the tumor growth every 3 days using vernier caliper, and calculate the tumor volume using the formula: $\text{length} \times \text{width}^2 \times 0.5$. Reduced tumor growth in vivo in mice due to a specific gene knockdown shows the role of the gene in tumor growth.

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⁵In some cases human cancer cell lines may not form tumor in athymic nude mice. In such cases injecting the cells with Matrigel might help. Additionally, using severe combined immunodeficiency (NOD-SCID) mouse model may increase the chance of tumor formation.

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