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A convenient cell culture model for CML acquired resistance through BCR-ABL mutations

Zhiqiang Wang and WenYong Chen

Department of Cancer Biology, Beckman Research Institute, City of Hope, Duarte, CA 91010

Summary

Tyrosine kinase inhibitors (TKIs) are the effective treatments for chronic myeloid leukemia (CML). However, clinical resistance to TKIs that leads to patient relapse remains a challenge. Acquisition of BCR-ABL mutations is crucial in the resistance but the underlying molecular mechanisms are poorly understood. Here we describe a cell culture model for CML acquired resistance in which blast crisis CML cells undergo initial apoptosis upon treatment with therapeutically effective doses of TKIs, but the cells re-grow quickly with development of resistance through BCR-ABL mutations. This model mimics the clinical process of acquisition of BCR-ABL mutations and will be an important tool to dissect molecular mechanisms of CML drug resistance and to explore strategies to overcome resistance.

Keywords

Chronic Myeloid Leukemia; Acquired Resistance; BCR-ABL; Mutation; Colony Formation Assay

1. Introduction

CML is a type of hematological malignancy resulting from BCR-ABL oncogenic transformation of hematopoietic stem cells. The potent ABL tyrosine kinase inhibitors are the effective treatments for the disease, which include imatinib (Gleevec, STI-571), Dasatinib (Sprycel), Nilotinib (Tasigna) and Ponatinib (Iclusig) etc. However, clinical resistance to the drugs and toxic side effects associated with newer/more potent drugs limit the power of the drugs, and the relapse particularly in advanced phases of the disease remains a significant challenge. The resistance is primarily mediated by BCR-ABL mutation and/or gene amplification (1). There are dozens of BCR-ABL mutations identified in CML patients, which cause resistance to TKIs to various degrees (1–3). Among them, T315I mutation is resistant to most of TKIs in clinic and the most difficult to treat (4–6). To explore the mechanisms of BCR-ABL mutation acquisition and resistance, and to facilitate development of new therapeutic strategies to overcome the resistance, a proper model system is needed.

Traditionally, resistant CML cell lines are generated by exposing cells to gradually increasing concentrations of TKIs. However, the resulting resistant cells harbor only *BCR-ABL* gene amplification but not mutations (7), in contrast to what is seen in many patients. Ectopically expressing mutant BCR-ABL cDNA in non-CML cell lines allows clinically-relevant or novel BCR-ABL mutations to render resistance to imatinib in these cells (4–6,8–13). However the mutagenesis process is ignored in such settings. Various BCR-ABL mutations can also occur in primary cultured CML cells in the growth-factor-supplemented medium (14,15); however, the pre-existing mutant cells in the primary samples can't be excluded and sometimes are difficult to track.

Here we describe a convenient cell culture model for CML TKI resistance using a blast crisis CML cell line KCL-22 cells (16). The cells will undergo initial apoptosis upon TKIs treatment, but will re-grow after two weeks with development of resistance through T315I BCR-ABL mutation. This model is simple, tractable and reproducible. The pre-existing BCR-ABL mutations are not required for the resistance in this model.

2. Materials

1. KCL-22 cell line was purchased from German Collection of Cell Cultures (DSMZ), Braunschweig, Germany. It was established from the pleural effusion of a 32-year-old woman with Philadelphia chromosome-positive CML in blast crisis phase. It contains p210 BCR-ABL fusion gene and a p53 mutation (deletion).
2. RPMI cell culture medium.
3. Penicillin Streptomycin (P/S), cell culture grade.
4. Characterized Fetal Bovine Serum (FBS) (Hyclone). The quality of this serum is essential to ensure the success and reproducibility of the model. Alternative serum will need to be tested and compared to Hyclone serum (See note 1).
5. Trypan blue solution.
6. Imatinib (STI-571), Dasatinib and Nilotinib: LC Laboratories.
7. Hematocytometer.
8. 100 mm culture dishes, 24- and 6-well plates.
9. 0.005% Crystal Violet solution prepared in 10% ethanol.
10. High fidelity DNA polymerase: PfuUltra HF DNA Polymerase (Agilent).
11. Liquid culture medium: RPMI + 10%FBS + 1x P/S

¹Serum used for culture is an important factor affecting the assay. While Hyclone characterized FBS provides the most consistent results, its cost becomes increasingly high. We typically screened alternative brands and batches of FBS and compared side by side with Hyclone characterized FBS. The results can vary significantly from brand to brand and sometimes batch to batch, suggesting that certain growth factors in serum affect the mutagenesis. It is recommended to use the same batch of serum for the experiments if possible. In addition, the quality of FBS should be re-examined if it is stored over a long period (e.g. one year).

12. 2× RPMI medium: dissolve one package of RPMI powder (Gibco) in 500 mL ddH₂O, add 2 g NaHCO₃, adjust PH to 7.05, filter the solution through 0.2 µM filter. Store at 4 °C.
13. 2× soft agar medium: Freshly prepared with 2X RPMI + 20% FBS + 2× P/S
14. 2× bottom layer agarose: 1.2% low gelling temperature agarose (cell culture grade, Sigma) in ddH₂O.
15. 2× top layer agarose: 0.7% low gelling temperature agarose in ddH₂O. Both agarose solutions should be autoclaved at 120 °C for 20 min and store at 4 °C.
16. RNA Purification Kit, Gel Extraction Kit and Miniprep Kit are from Qiagen.
17. TA Cloning Kit and CloneJET PCR Cloning Kit are from Life Technologies.

3. Methods

Carry out all procedures in biological safety cabinet unless specified otherwise.

3.1 KCL-22 cell culture maintenance

Freshly thawed cells are seeded at a density of 0.5 million per mL in RPMI culture medium. The cells grow fast and need to be split 1:5 every 2–3 days to maintain the density at 0.2–1 million per mL. It is very important not to overgrow KCL-22 cells (the cell density > 2 million/mL) and it is also important to record cell passage number when they are split. (*See note 2*)

3.2 Resistance assay in liquid culture

1. Harvest KCL-22 cells from one 100 mm dish and spin down the cells at 300 ×g for 3 min.
2. Re-suspend the cells in 1× RPMI culture medium, count the cells and adjust the cell density to 0.5 million per mL.
3. Seed 1 ml cell suspension (0.5 million cells) per well in 24-well plates. (*See note 3*)
4. Treat the cells with different concentrations of test drug or drug combinations. (e.g. STI-571). Each group should have duplicate or triplicate wells.

²KCL-22 overgrowth may affect resistance assay dramatically and may lead to complete failure of the assay. We also noticed that cell passages affect the assay. Cells with passage later than P30 (P1 is designated for the cells after initial thawing and plating of the frozen cells from the vendor) have less resistant colonies and a delayed relapse. Cells with lower passages give better readouts.

³We noticed that the wells around the edge of plates sometimes gave less consistent cell relapse results if the culture lasted for longer than 2 weeks. To achieve more consistent results, it is recommended to seed cells in the center wells of a plate, and fill the wells at the edge with sterile saline or water. If the edge wells should be used, they should be coupled with the center wells as replicates.

5. Maintain the cells in culture without changing medium or drugs unless the test drug is not stable in culture.
6. Count viable cells at different time (day) points. At specified time points, the cells are mixed by gently pipetting up and down with a 1 mL pipette for several times and a 10 μ L aliquot of cell suspension is removed and mixed with 10 μ L Trypan blue solution. Load 10 μ L mixed sample onto a hemacytometer and count the viable cells (trypan blue exclusive cells). After two weeks' culture, the medium volume would significantly decrease and fresh drug-free medium is supplied to the cells to restore that to the original volume for prolonged culture.
7. Typically the cell number would decrease in the first week and the resistant cells would begin to appear in small clusters in non-agitated wells after a week and re-grow back in about 10 days. Once the re-grown cell density exceeds 1.0 million per mL, the growth curve can be terminated. The relapsed cells can be collected to extract DNA, RNA or protein for further analysis. A typical cell growth curve is shown in Figure 1.

3.3 Resistant colony formation assay in soft agar

1. Prepare 2 $\times$ RPMI soft agar medium freshly as in Material #12. Warm the medium to 37 $^{\circ}$ C in water bath.
2. Melt 2x agarose solutions completely by heating with a microwave oven. Leave the bottle lid open during heating. Put the agarose solution in 37 $^{\circ}$ C water bath to cool down to 37 $^{\circ}$ C for at least half an hour. (*See note 4*)
3. Bottom layer soft agar preparation: For each group (3 wells out of a 6-well plate), take 3.5 mL 2 $\times$ RPMI complete medium into a 15 mL conical tube, add the tested drug (*See note 5*) into the medium to reach 2 $\times$ desired concentration, and then mix well with 3.5 mL 1.2% agarose solution at 1:1 ratio. Aliquote 2 mL mixed solution into each well and keep the plate at room temperature until the solution gelatinizes (It would take about half an hour).
4. Top layer soft agar preparation: For each group (3 wells out of a 6-well plate), take 3.5 mL 2 $\times$ RPMI complete medium into a 15 mL conical tube, add the tested drug into medium to reach 2 $\times$ desired concentration, and then mix well with 3.5 mL 0.7% agarose solution at 1:1 ratio. Keep the mixture in 37 $^{\circ}$ C water bath until use.
5. Cell preparation: Harvest and count the KCL-22 cells. Put 3.5 million cells into a 15 mL conical tube. Spin down the cells and get rid of the supernatant. Re-suspend the cell pellet in 50 μ L RPMI culture medium

⁴Hot medium ($>37^{\circ}\text{C}$) would hurt cells and cause failure of the colony formation assay.

⁵If single TKI is used, the following concentrations are typically applied: Imatinib 1–10 μM , Dasatinib 0.1–1 μM , Nilotinib 0.5–10 μM . Lower TKI concentrations would be used if they are in combination with other drugs.

with a pipette and gently mix cells by pipetting up and down for a few times.

6. Add 7 mL top layer soft agar solution (From step 4) to the cells and pipet the solution to fully mix them. Gently seed 2 mL suspension solution into each well on top of the bottom layer of agarose. Thus 1 million cells per well are seeded.
7. For control wells of colony formation without drugs, five hundred KCL-22 cells per well will be similarly mixed with top layer soft agar solution and seeded.
8. Keep the plates at room temperature for at least half hour to allow the top layer gel solidifying.
9. Put the plates back into the incubator and culture for 3 weeks. (To clone or amplify soft agar colonies for further analysis, skip to step 12.)
10. Colony staining: 2 mL 0.005% Crystal Violet solution is added into each well and incubate for 1 hour at room temperature. Then the staining solution is removed and the background is de-stained by ddH₂O rinse for 3 times.
11. Count colony number with aid of a microscope. (*See note 6*).
12. If further analysis of colonies such as DNA sequencing is necessary, individual colonies can be plucked from unstained wells and expanded in liquid culture to enough number of cells for such analysis.

3.4 Sequencing analysis

3.4.1 cDNA sequencing

1. Extract mRNA from the relapsed cells and do reverse transcription to obtain cDNA by standard methods.
2. Amplify the ABL kinase domain by PCR with a high fidelity DNA polymerase using a forward primer 5'-GCGCAACAAGCCCACTGTCTATGG and reverse primer 5'-GCCAGGCTCTCGGGTGCAGTCC that amplify the 579 bp ABL kinase domain. (*See note 7*)
3. Clean the PCR product with a PCR purification kit. Alternatively, run the PCR product on an agarose gel and purify the band using a gel extraction kit.
4. Sequence the product to determine the mutation.

⁶This cell model can be used to test resistance through other gene mutations, e.g. 6-thioguanine resistance through HPRT mutation (16).

⁷The interested regions may be amplified by PCR using different primers and sent for sequencing. The hot mutation spots should be covered.

5. If the sequencing results indicate overlaying peaks (Fig 2A), it suggests potential mutations. PCR products will then be cloned into pCR2.1 vector with a TA Cloning Kit or blunt-end ligated into a vector with CloneJET PCR Cloning Kit, followed by transformation of bacterial competent cells. We typically sequence at least ten bacterial clones for each treatment group to determine the mutation rate in a mixture setting (Fig 2B). (*See note 8*)

3.4.2 Genomic DNA sequencing—To further determine the mutations, genomic DNA can also be used for amplification for sequencing.

1. Extract genomic DNA from the relapsed cells.
2. Amplify the ABL kinase domain by PCR using genomic DNA as templates with the intron primers: 5'-GAGCCACGTGTTGAAGTCCT-3' and 5'-TTTGTAAGGCTGCCCCGGC-3' that span ABL exon 6 for T315I mutation, and intron primers 5'-GCCTGTCTCTGTGGGCTGAAG-3' and 5'-TAATGCCAGCAGACGCCTTG-3' that span ABL exon 4 for E255K and Y253H mutations.
3. Clean the PCR product as in step 3 in section 3.4.1. Submit the samples for Sanger sequencing.
4. If the sequence results show overlaying peaks (Fig 2A), PCR products will be subcloned and sequenced as in step 5 in section 3.4.1. Typically, about 30% to 50% of clones have mutations since KCL-22 cells harbors one wild-type allele of c-ABL gene that is also amplified for sequencing.
5. Primers for other parts of BCR-ABL or other genes: For oligomerization domain, we used forward primer 5'-GAGTGGGCGGGCATTGTTC and reverse primer 5'-GGGACTTTTTCGCTCCATCT. For sequencing BCR-ABL SH3/2 domain, we used primers described previously (11).

3.5 Extended mutation analysis for ectopically expressed BCR-ABL cDNA

The BCR-ABL mutagenesis is a locus-dependent process and the mutagenesis potential between the endogenous BCR-ABL locus and exogenously integrated BCR-ABL is quite different (16). To analyze such difference, we used KCL-22 cells ectopically expressing BCR-ABL by retroviral expression system. When resistant cells develop as in 3.2 or 3.3, the endogenous BCR-ABL locus and integrated BCR-ABL cDNA can be PCR amplified from genomic templates and distinguished with intron/intron primer pair (as in step 2 in section 3.4.2.) and exon/exon primer pair (as in step 2 in section 3.4.1), respectively. To amplify the exogenous BCR-ABL template more efficiently, we also use another reverse primer 5'-TAGTCCAGGAGTTCCCGTAG to pair with the same exon forward primer, which yields a 321 bp PCR product.

⁸KCL-22 cells carry one copy of wild type ABL gene in addition to BCR-ABL fusion gene. Therefore the sequencing results of PCR products usually have mixed signals and need to be examined manually.

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Figure 1.
Representative growth and relapse curves of KCL-22 cells in response to TKIs. IM, imatinib; Das, dasatinib; Nil, nilotinib.

Figure 2. Sequencing analysis of ABL codons. (A) Direct PCR product sequencing showed overlaying C/T peaks in relapsed cells. (B) Sequencing of bacterial clones of PCR products.