M. Chi, et al. [2011] Med. Hypotheses Res. **7**: 27–33.

HYPOTHESIS

A Dominant Genetic Mechanism for Acquired Imatinib-Resistance of Blast Crisis Chronic Myeloid Leukemia

Martin Chi§, Qiang Liu§, Linling Chen, Yafan Wang, WenYong Chen, Hongfeng Yuan, Tomas Radivoyevitch and Yun Yen*

Department of Molecular Pharmacology and Translational Research Core Laboratory (M.C., L.C., Y.W., Y.Y.), Department of Cancer Biology, City of Hope Beckman Research Institute and National Medical Center, Duarte, CA 91010 (W.Y.C.), Department of Epidemiology and Biostatistics, Case Western Reserve University, Cleveland, OH 44106, USA (T.R.)

Abstract. A dominant genetic mechanism is hypothesized and tested for acquired drug resistance of blast crisis chronic myeloid leukemia, which is initially sensitive to imatinib but shortly develops resistance due to genetic mutations. A cell line KCL-22 of blast crisis chronic myeloid leukemia is exemplified, of which each cell contained two copies of Philadelphia chromosome t(9;22) harboring the *BCR-ABL* fusion gene. When one of these two copies in an individual cell was mutated to 315I, the cell was sufficient to become imatinib resistant, suggesting a dominant genetic mechanism. This mechanism was also supported by mathematical simulation of cell growth kinetics. Thus, the copy number of the susceptible gene and the copy number of causative mutations are correlated with the resistance phenomenon. Clinically, the copy number of a susceptible diploid or multi-ploid gene due to cell fusion, gain of chromosomes, or gene amplification plays important roles in drug resistance and serves as markers to guide therapeutic decisions.

* **Correspondence:** Dr. Yun Yen, Department of Molecular Pharmacology, City of Hope, Arnold & Mabel Beckman Center Building, 1500 East Duarte Blvd, Duarte, CA 91010, USA. TEL: 626-256-4673 (ext. 65707). E-MAIL: yyen@coh.org

§ These two authors contributed almost equally.

Received on 04-10-2008; accepted on 05-18-2008.

1. Introduction

Targeted therapeutics, such as the *BCR-ABL* tyrosine kinase inhibitor imatinib for chronic myeloid leukemia (CML), are widely used, but acquired drug resistance limits their broader success [1]. Acquired drug resistance is a phenomenon in which, after a drug is given, cancer cells still accumulate in number over time because of acquired mutations. Needless to say, mutations can also be acquired before therapy.

CML is characterized by the Philadelphia (Ph) chromosome, which results from the t(9;22)(q34;q11) translocation [2]. The molecular consequence of this translocation generates the *BCR-ABL* oncogene that encodes the chimeric *BCR-ABL* tyrosine kinase. Imatinib, the *BCR-ABL* inhibitor, leads to complete cytogenetic responses and infrequent relapses in most chronic phase CML patients, but unfortunately is inefficacious in advanced phase patients [3]. T315I as well as many other *BCR-ABL* resistant mutations have been identified in relapsed CML patients [3-5].

A dominant genetic mechanism is hypothesized and tested for acquired imatinib resistance in sensitive blast crisis KCL-22 cell line where each cell contained two copies of the *BCR-ABL* fusion gene (FIG S1, SUPPLEMENTARY DATA) [6]. When one of these two copies in an individual sensitive cell was mutated to 315I, the cell, now termed as KCL-22M, was sufficient to become imatinib resistant, suggesting a dominant genetic mechanism. Thus, the copy number of the susceptible gene, the copy number of the causative mutation, and the resistance are correlated. Clinically, through this dominant mechanism, drug resistance arises earlier and more frequently because of multiple copies of a susceptible gene due to cell fusion, gain of chromosomes, or gene amplification fusion [7,8].

2. Materials and Methods

2.1. Sanger sequencing analysis of genomic DNA clones from individual resistant cells

To determine whether KCL-22M resistant cells contain either one or two copies of T315I mutation per cell, an intron 5 primer 5′-GAGCCACGTGTTGAAGTCCT-3′ and an exon 6 primers 5′-TTTGTAAAAGGCTGCCCGGC-3′ were designed to span exon 6 within the *ABL* kinase domain. In this design, two copies of the fusion *BCR-ABL* gene and one copy from the *ABL* gene per cell were amplified by PCR. After purified by Qiagen MinElute PCR purification kit, the PCR product was sequenced with each primer using ABI 3730 fluorescent DNA sequencer and BigDye terminator chemistry V3.1 (Applied Biosystems). Sequencher software (Gene Codes) was used to identify the mutation on chromatograph.

2.2. Resistance assay

Half a million KCL-22 sensitive cells or 1000 KCL-22M resistant cells were seeded in 1 ml of RPMI 1640 medium with 10% fetal bovine serum (Hyclone, SH30071.03) per well in triplicate and treated with 2.5, 5 or 10 μM imatinib (STI-571) [6]. Aliquots of cells were removed at given time points, the number of cells was counted on a hematocytometer, and cell viability was assessed by trypan blue exclusion.

2.3. Soft agar colony formation assay

For colony formation, a standard two-layer soft agar culture was used at 2.5, 5 or 10 μM imatinib [6]. One million KCL-22 sensitive cells were seeded per well in triplicate and incubated for 3 weeks. Plates were then stained with 0.005% Crystal Violet for 1 hour, and the number of colonies was scored by microscope.

2.4. Statistical analysis

Mathematical models corresponding to genetic MECHANISM B and C as depicted in FIG 2 were fitted to the data. The Akaike Information

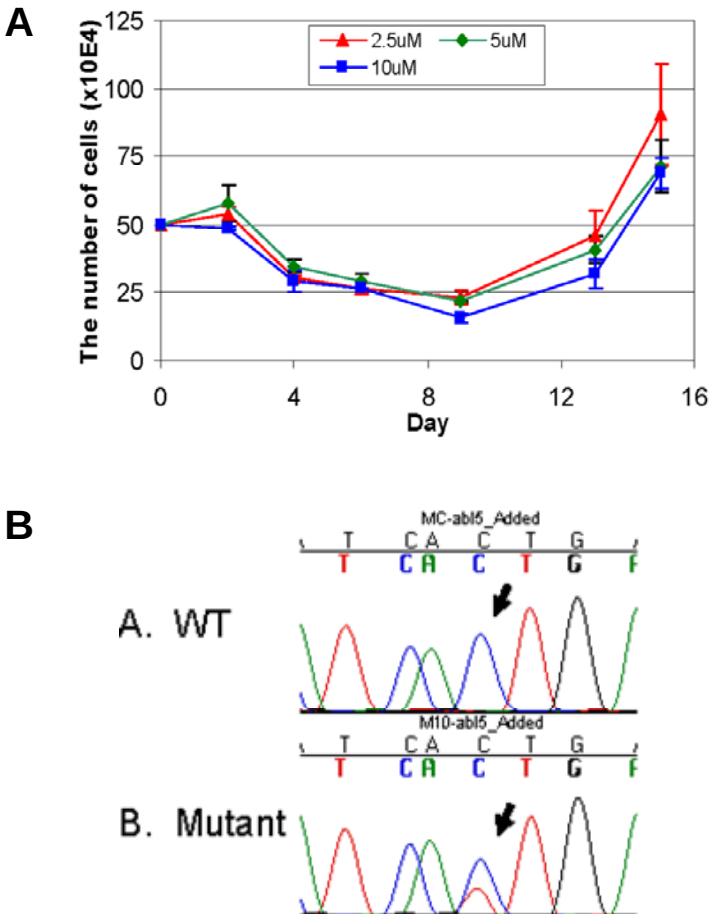

Figure 1. Identification of imatinib resistance. A. Observed cell growth kinetics with imatinib. X-axis represents time with units in days. Y-axis shows the number of viable cells averaged in triplicate with standard deviation. In the earlier days of the experiment, virtually all the cells were KCL-22 cells, but in later days most, if not all, cells were KCL-22M cells, each of which contained a copy of the T315I mutation. B. Sanger sequencing analysis of genomic DNA from individual resistant clonal cells. In the example chromatograph shown, two copies of the fusion *BCR-ABL* gene and one copy from the *ABL* gene of each cell were amplified by PCR and sequenced by Sanger sequencing with the downstream primer. On the chromatograph, the mutated T peak is 50% as high as that of the wild-type C peak, suggesting that only one copy of the fusion *BCR-ABL* gene contain the T315I mutation per cell.

Criterion [9], which penalizes models for additional parameters that are not sufficiently effective in improving the fit, was used to select the best model, and thus support the underlying hypothesis. Model differences in goodness of fit largely occurred when disease recurrence became obvious at an early recurrence day 13.

3. Results and Discussion

3.1. Acquired imatinib resistance of blast crisis KCL-22 cell line

Chronic phase CML typically harbors one copy of the *BCR-ABL* fusion gene per cell. When the single copy is mutated to 315I, the individual cell becomes resistant to imatinib [3-5,10].

However, advanced phase CML may contain two copies of the *BCR-ABL* fusion gene per cell, such as in the case of blast crisis KCL-22 cell line (FIG S1, SUPPLEMENTAL DATA). When treated with 2.5, 5 or 10 μM imatinib, equivalent to the effective concentrations in clinical treatments [11], KCL-22 sensitive cells underwent initial apoptosis but relapsed after eight to nine days due to emergence of KCL-22M resistant cells (FIG 1A).

Using PCR and Sanger sequencing, we showed that KCL-22M resistant cells carried T315I mutation in *BCR-ABL* kinase domain [6]. To determine whether each resistant cell contained one or two copies of T315I mutation, we further sequenced 20 genomic DNA clones from individual resistant cells and found that all the 20 resistant clones carried only one copy of T315I mutation per cell (FIG 1B). Resistant clones that contained two copies of T315I mutation per cell were not identified, presumably due to its

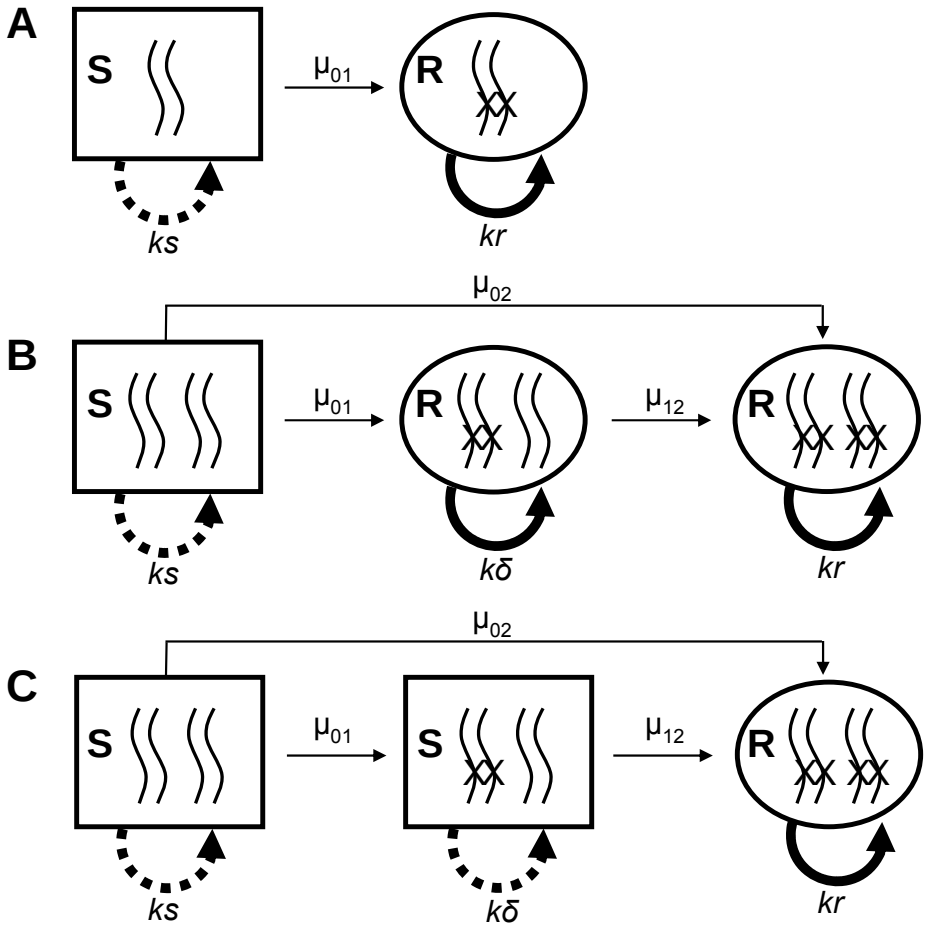

Figure 2. **Genetic mechanisms of drug resistance: occurence and development. A. Single copy mechanism. Each sensitive or resistant cell contains only a single copy of any susceptible gene, such as the *BCR-ABL* fusion gene. Two distinct types of tumor cells exist: sensitive (squared) and resistant (circled). The circular arrow represents the net growth, the values of which may be positive (solid line) or negative (dotted line). Each pair of curved lines represents one copy of a gene and a cross represents one copy of a mutant gene. The straight arrow indicates occurence of mutations. The mutation event may acompany a conversion from a sensitive cell into a resistant cell. B. Dominant mechanism. Each sensitive or resistant cell contains two copies of a susceptible gene. One mutant copy is sufficient for resistance. C. Recessive mechanism. Each sensitive or resistant cell contains two copies of a susceptible gene. Two mutant copies are required for resistance.**

low frequency. Thus, our result suggests that only one mutant copy be sufficient for an individual sensitive cell to become resistant and that most, if not all, resistant cells contained only one mutant copy per cell.

In addition, no other mutations were identified in the kinase domain as well as other functionally important oligomerization and SH3/2 domains of the *BCR-ABL* gene in KCL-22M resistant cells [6]. Furthermore, KCL-22M resistant cells shared many similar characteristics to KCL-22 sensitive cells (TABLE S1) [6].

3.2. Genetic mechanisms of acquired drug resistance

Acquired drug resistance is often caused by a point mutation of the drug targeted gene. It is observed that: 1) resistant tumor cells are distinct from sensitive cells by their net cell growth rates at a given drug concentration and 2) an acquired mutation may convert a sensitive cell into a

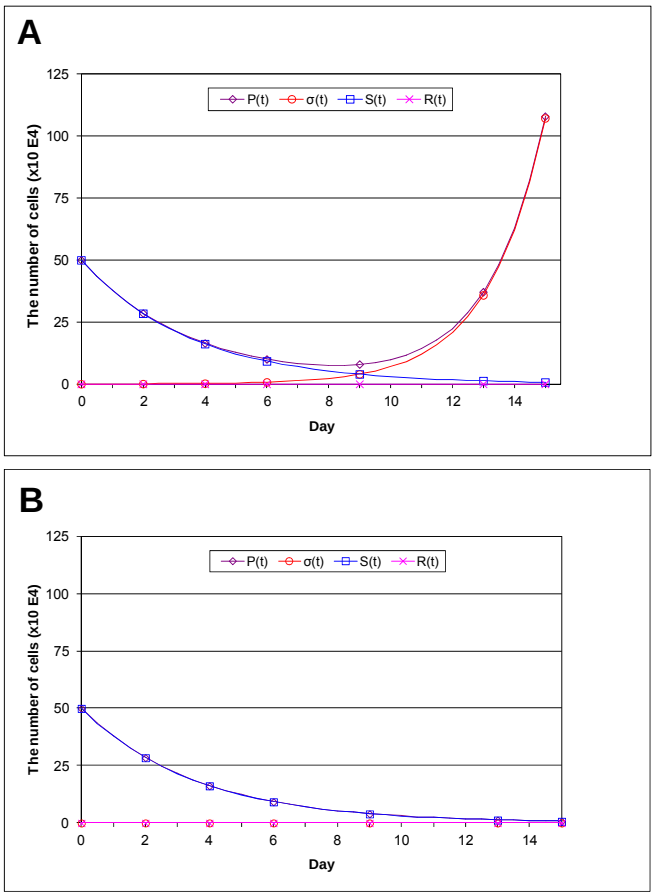

Figure 3. **Simulated cell growth kinetics of imatinib resistance. A. Simulated data based on *mechanism B*. At 2.5 μM imatinib, the number of initial KCL-22 sensitive cells having zero mutant copies per cell, S_0, was 500,000 and its growth rate, k_s, was -0.245. The numbers of initial KCL-22M resistant cells having 1 or 2 mutant copies per cell, δ_0 and R_0, were zero and their growth rates, k_δ and k_r, were 0.730. Transfer rates, μ_{01}, μ_{02}, μ_{12}, as well as μ_{00} and μ_{11} are detailed in TABLE S3. At given days, the numbers of sensitive and resistant cells having 0, 1 and 2 mutant copies per cell, $S(t)$, $\delta(t)$ and $R(t)$, were calculated according to Eq s1. Similar patterns were obtained when 120 resistant cells were assumed to pre-exist (r_0 =0, δ_0 = 120). B. Simulated data based on *mechanism C*. At 2.5 μM imatinib, the number of initial sensitive cells having zero and 1 mutant copy per cell, S_0 and δ_0, was 500,000 and zero, respectively. Their growth rates, k_s and k_δ, were -0.245. The numbers of initial KCL-22M resistant cells having 2 mutant copies per cell, R_0, was zero and its growth rate, k_r, was 0.730. Similar patterns were obtained when 120 resistant cells were assumed to pre-exist (r_0 =0, δ_0 = 120).**

resistant cell. Accordingly, several mechanisms of acquired drug resistance are depicted in FIG 2. In the simplest case, shown in FIG 2A, only one copy of a susceptible gene is present in a tumor cell, which is equivalent to a haploid genome. When this single copy is mutated, the cell becomes resistant. For instance, in chronic phase CML patients, when the single copy of the *BCR-ABL* fusion gene is mutated to 315I, the cell becomes resistant to imatinib [3-5,10].

However, in advanced phase, a CML cell may contain two copies of *BCR-ABL*, such as in the case of blast crisis KCL-22 cell line. This leads to two other potential mechanisms (FIG 2B, 2C). With MECHANISM B, one mutant copy is sufficient for resistance, which is similar to a dominant inherited trait of a diploid genome. On the other hand, with MECHANISM C, two mutant copies are required for resistance, which is similar to a recessive inherited trait of a diploid genome.

3.3. Simulated cell growth kinetics of blast crisis KCL-22 cell line

To quantitatively discriminate between MECHANISM B and C (FIG 2B, 2C), mathematical models were developed for simulation. We assumed: 1) that under a given drug concentration, KCL-22 sensitive cells and KCL-22M resistant cells grow at constant but different rates during the exponential growth phase; and 2) that the rate of T315I mutation is constant.

Growth and mutation rates were measured from resistance assays and soft agar colony formation assays, respectively (TABLE S2, SUPPLEMENTARY DATA). In addition, in order to show transfer rates in the network, a probability

matrix of μ_{01}, μ_{02}, μ_{12}, as well as μ_{00} and μ_{11} was introduced from conversion of the rate of T315I mutation observed on soft agar colony formation assay. For instance, μ_{01} is the transfer rate from the sensitive cells with zero mutant copies to the resistant cells with one mutant copy (TABLE S3). Exponential growth functions (Eq. 1 in SUPPLEMENTARY DATA) were chosen because they fit the negative cell growth of KCL-22 sensitive cells the best.

Therefore, taking the experimental growth and mutation rates at 2.5, 5, or 10 μM imatinib, we simulated cell growth kinetics to reveal the underlying mechanism that is most consistent with our data (FIG 3A, 3B). Simulations were performed with and without pre-existing resistant cells.

At 2.5 μM imatinib, we tested whether the simulated cell growth patterns from MECHANISM B (FIG 3A) fit the observed kinetics in FIG 1A. On day 13, at an early stage of relapse, the number of viable cells was also compared between the experiment and simulation using a t-test (P = 0.27 and 0.37 without and with the pre-existing mutation, respectively), supporting MECHANISM B (FIG 2B). In contrast, the simulated cell growth patterns from MECHANISM C did not fit the data on day 13 (P = 0.016 both with and without the pre-existing mutation).

At higher concentrations of 5 or 10 μM imatinib, the simulated cell growth patterns from MECHANISM B fit the observed kinetics much better than those from MECHANISM C (on day 13, P > 0.02 and 0.03 with and without the pre-existing mutation with MECHANISM B *vs*. P < 0.01 and 0.01 with MECHANISM C). This result is compatible with that at a dose of 2.5 μM.

3.4. Simulated mutation rates of blast crisis KCL-22 cell line

Furthermore, the mutation rates were simulated from the experimental growth rates (TABLE 1). According to MECHANISM B, the simulated values are much closer to the observed mutation rates obtained from the colony formation assay. Again, the data supports MECHANISM B but not C.

3.5. Conclusions

A dominant genetic mechanism is hypothesized and tested for acquired imatinib resistance in blast crisis KCL-22 cell line. We showed that when one of the two copies of the *BCR-ABL* fusion gene was mutated to 315I, an individual cell was sufficient to become resistant. Thus, the copy number of the susceptible gene, the copy number of the causative mutation, and the resistance are correlated. Clinically, through this dominant mechanism, drug resistance arises earlier and more frequently because of multiple copies of a susceptible gene due to cell fusion, gain of chromosomes, or gene amplification fusion [7,8].

References

1. **Baccarani M, Cortes J, Pane F, Niederwieser D, Saglio G, Apperley J, Cervantes F, Deininger M, Gratwohl A, Guilhot F, et al.** [2009] Chronic myeloid leukemia: an update of concepts and management recommendations of European Leukemia Net. J Clin Oncol 27: 6041-6051.
2. **Quintas-Cardama A and Cortes J** [2009] Molecular biology of bcr-abl1-positive chronic myeloid leukemia. Blood 113: 1619-1630.
3. **Gorre ME, Mohammed M, Ellwood K, Hsu N, Paquette R, Rao PN and Sawyers CL** [2001] Clinical resistance to STI-571 cancer therapy caused by BCR-ABL gene mutation or amplification. Science 293: 876-880.
4. **Shah NP, Nicoll JM, Nagar B, Gorre ME, Paquette RL, Kuriyan J and Sawyers CL** [2002] Multiple BCR-ABL kinase domain mutations confer polyclonal resistance to the tyrosine kinase inhibitor imatinib (STI571) in chronic phase and blast crisis chronic myeloid leukemia. Cancer Cell 2: 117-125.
5. **Branford S, Rudzki Z, Walsh S, Grigg A, Arthur C, Taylor K, Herrmann R, Lynch KP and Hughes TP** [2002] High frequency of point mutations clustered within the adenosine triphosphate-binding region of BCR/ABL in patients with chronic myeloid leukemia or Ph-positive acute lymphoblastic leukemia who develop imatinib (STI571) resistance. Blood 99: 3472-3475.
6. **Yuan H, Wang Z, Gao C, Chen W, Huang Q, Yee JK, Bhatia R and Chen W** [2010]. BCR-ABL gene expression is required for its mutations in a novel KCL-22 cell

culture model for acquired resistance of chronic myelogenous leukemia. J Biol Chem 285: 5085-5096.
7. **Pawelek JM** [2005] Tumour-cell fusion as a source of myeloid traits in cancer. Lancet Oncol 6: 988-993.
8. **Margolis RL** [2005] Tetraploidy and tumor development. Cancer Cell 8: 353-354.
9. **Akaike H** [1974] A new look at the statistical model identification. IEEE Transactions on Automatic Control 19: 716-723.
10. **Michor F, Hughes TP, Iwasa Y, Branford S, Shah NP, Sawyers CL and Nowak MA** [2005] Dynamics of chronic myeloid leukaemia. Nature 435: 1267-1270.
11. **Peng B, Lloyd P and Schran H** [2005] Clinical pharmacokinetics of imatinib. Clin Pharmacokinetics 44: 879-894.

Conflict of interest statement: The authors have no conflict of interest to declare.