Video Article

Looking for Driver Pathways of Acquired Resistance to Targeted Therapy: Drug Resistant Subclone Generation and Sensitivity Restoring by Gene Knock-down

Chiara Arienti¹, Sara Pignatta¹, Michele Zanoni¹, Michela Cortesi¹, Alice Zamagni¹, Filippo Piccinini², Anna Tesei¹

¹Biosciences Laboratory, Istituto Scientifico Romagnolo per lo Studio e la Cura dei Tumori (IRST) IRCCS

²Istituto Scientifico Romagnolo per lo Studio e la Cura dei Tumori (IRST) IRCCS

Correspondence to: Anna Tesei at anna.tesei@irst.emr.it

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Abstract

The past two decades have seen a shift from cytotoxic drugs to targeted therapy in medical oncology. Although targeted therapeutic agents have shown more impressive clinical efficacy and minimized adverse effects than traditional treatments, drug resistance has become the main limitation to their benefits. Several preclinical *in vitro/in vivo* models of acquired resistance to targeted agents in clinical practice have been developed mainly by using two strategies: i) genetic manipulation for modeling genotypes of acquired resistance, and ii) *in vitro/in vivo* selection of resistant models. In the present work, we propose a unifying framework, for investigating the underlying mechanisms responsible for acquired resistance to targeted therapeutic agents, starting from the generation of drug-resistant cellular subclones to the description of silencing procedures used for restoring the sensitivity to the inhibitor. This simple time- and cost-effective approach is widely applicable, and could be easily extended to investigate resistance mechanisms to other targeted therapeutic drugs in different tumor histotypes.

Video Link

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Introduction

Following up on the crucial discoveries in molecular and cellular biology, a number of novel synthesized anticancer molecules have been developed to selectively target oncogenic signaling pathways in a broad array of tumor types. In particular, two categories of targeted therapeutic agents with unique properties in oncology, namely synthetic chemical compounds and recombinant monoclonal antibodies, are known to have achieved clinical successes and dramatically altered cancer care in recent decades^{1,2,3}.

Nevertheless, despite their impressive initial response to treatment, the majority of cancer patients have developed drug resistance to all targeted therapeutic agents, both monoclonal antibodies and kinase inhibitors. As a consequence, drug resistance, the major hurdle for classic anti-cancer medicines⁴, is still a great challenge for emerging targeted therapies^{5,6}.

Resistance to targeted therapy may be intrinsic (*i.e.*, primary) or acquired (*i.e.*, secondary). Intrinsic resistance describes a *de novo* lack of response to therapy, whereas secondary resistance occurs after a period of response to drug treatment⁷. The latter is caused by outbreaks of small cohorts of tumor cells within the bulk of the primitive tumor or nestled in distal cryptic anatomical niches, exhibiting up to 90% resistance to one or more targeted therapeutic agents. Molecular mechanisms underlying a resistance development to targeted therapy mainly result from target gene mutations and redundant activation of other pro-survival signaling pathways, whose understanding is still far from complete⁸.

In this work, we proposed a time- and cost-effective approach to investigate *in vitro* mechanisms of acquired resistance to targeted therapeutic agents, starting from the generation of drug-resistant cellular subclones to the description of silencing procedures used for restoring the sensitivity to the inhibitor, a crucial tool used for testing and validating the working hypotheses. In particular, our approach was used to investigate, in gastric cancer, the resistance mechanisms to trastuzumab (*e.g.*, Herceptin), a humanized monoclonal antibody targeting the extracellular domain of HER2 protein⁹. Trastuzumab + chemotherapy is widely accepted as the standard first-line treatment for HER2-positive metastatic breast cancer. Thanks to recent preclinical studies showing that anti-HER2 therapies have significant activity in both *in vitro* and *in vivo* HER2-positive gastric cancer models^{10,11}, molecular drugs targeting HER2 have been extensively examined in clinical trials, some of which are still ongoing, on patients with gastroesophageal cancer^{12,13,14,15}.

These studies have emphasized the rising number of patients experiencing resistance to trastuzumab¹⁶, similarly to what is observed for other targeted therapeutic inhibitors. In particular, the response rate of trastuzumab was 47%, and the median overall survival for patients on trastuzumab + chemotherapy was only 2.7 months longer than for patients on chemotherapy alone¹⁷. This suggested that while primary trastuzumab resistance is prevalent, secondary trastuzumab resistance is inevitable. For this reason, there is urgent need to clarify the

mechanisms causing HER2-targeted therapy resistance in gastric cancer, which is even more problematic given the intra-tumoral genetic heterogeneity of this neoplasia¹⁸.

In addition, mechanisms of resistance to trastuzumab in gastric cancer have proven challenging to explain, partially due to the difficulty in obtaining reliable preclinical models representative of this condition. Oshima *et al.* reported an *in vivo* selection method of trastuzumab-resistant gastric cancer cell lines, consisting of a repeated culture of a small residual peritoneal metastasis after treatment with trastuzumab¹⁹. However, the need of an enclosure, of specific animal handling expertise, and of the approval of the Animal Ethics Committee contributed to making it a time- and cost-consuming method. The approach we describe in this work is simple and widely applicable, and could be easily extended to investigate the resistance mechanisms to other therapeutic drugs in different tumor histotypes²⁰.

Protocol

NOTE: This protocol has been specifically adjusted for the analysis of the mechanisms underlying acquired resistance to trastuzumab of HER-positive gastric cancer cell lines. All laboratory instruments needed are reported in the **Table of Materials**.

1. Generation of Targeted Therapy Resistant Subclones

NOTE: This is the most critical and difficult to standardize step in the protocol due to the fact that different cell lines may exhibit different sensitivities to the targeted therapeutic inhibitor independently from their tumor histotype or drug concentration used.

1. Culture NCI-N87 cell line in RPMI medium supplemented with fetal calf serum (10%) and glutamine (2 mM, 1%), as a monolayer at 37 °C, and seed it onto 25 cm² culture flasks.
2. Add to the culture medium in each flask 10 µg/mL of trastuzumab as the starting dose. Expose the cells to the starting dose until they resume growing.
NOTE: The starting dose of the targeted therapy inhibitor should be 10-fold lower than its plasma peak concentration. Plasma peak concentration is the highest level of drug concentration detected in patient blood usually following multiple doses of standard therapy. This value can be retrieved from studies on pharmacokinetics.
3. Monitor cell growth with the trypan blue exclusion method; when cell growth is resumed (typically after a period of two to three weeks), expose cells to a 10-fold higher dose concentration of trastuzumab.
4. Expose cells to a gradually increasing dose of trastuzumab (from 100 µg/mL to 400 µg/mL) until cells keep growing (after about 2 weeks) despite the presence of a high drug concentration (at least 2.5 - 4-fold higher than the plasma peak).
5. **Perform a clonogenic assay to evaluate the level of resistance to targeted therapy at every change in the concentration of the target therapy inhibitor in the culture medium, and define relative resistance IC₅₀ index (RR IC₅₀) as follows.**
 1. Seed 500 cells in 24 multi-well culture plates.
NOTE: 5 wells must be prepared for each condition set.
 2. Expose cells to increasing target therapy inhibitor (trastuzumab) concentrations from 100 µg/mL up to 400 µg/mL.
 3. Incubate cells in a CO₂ incubator at 37 °C for 15 days.
 4. After incubation, fix and stain samples as follows.
 1. Remove medium and rinse cells with 10 mL 1x PBS. Remove 1x PBS and add 2 - 3 mL of fixation solution (acetic acid:methanol, 1:7, (vol/vol)). Remove the fixation solution and add 0.5% crystal violet solution. Incubate at room temperature for 2 h
 2. Remove the crystal violet solution carefully by aspirating with a pipette and immerse plates in tap water to rinse off any crystal violet solution residues. Air-dry at room temperature for up to 2 days.
 5. Count colonies of more than 50 cells using an inverted microscope (4X magnification).
NOTE: Two independent observers are needed for this.
 6. Calculate RR IC₅₀ as the ratio between the IC₅₀ value of target-therapy-resistant subclone cells and the value of the original/naïve cells.

2. Validation Protocol for the Candidate Targeted Therapy Resistance Gene (R-gene)

NOTE: After the characterization of gene expression, signatures associated with the acquired resistance to target therapy and any candidate gene as driver of resistance will be investigated through: a) RT-PCR, and b) Western blot analysis.

1. **Real-time RT-PCR**
 1. Extract total RNA from cell lines using acid guanidinium thiocyanate-phenol-chloroform mixture (see **Table of Materials**).
 2. Perform reverse transcription (RT) reactions using random hexamer primers and set the thermal cycler as follows: priming at 25 °C for 5 min, RT at 46 °C for 20 min, and RT inactivation at 95 °C for 1 min.
 3. Use 400 ng of total RNA for a 20 µL reaction. Prepare the reaction mixture for sample as follows: 7 µL RNase free H₂O, 2 µL cDNA (10 ng/µL), 1 µL 20x fluorescent-labeled target-specific probe, and 10 µL 2x Mix containing buffer, dNTPs, and DNA polymerase (see **Table of Materials**).
 4. Add 20 µL of the reaction mixture to each well plate and run the following program: holding stage at 50 °C for 2 min, at 95 °C for 10 min (1 cycle); then 40 cycles at 95 °C for 15 s and at 60 °C for 1 min.
2. **Western blot**
 1. Recover cell suspensions by adding 2 mL of 0.05% trypsin/EDTA solution and allowing 2 - 3 min for trypsin to work.
 2. Add 2 volumes of medium containing 10% fetal bovine serum to neutralize trypsin (e.g., 2 mL of medium for each 1 mL of trypsin) and centrifuge at 1,200 x g for 5 min. Discard supernatant and wash cells with cold 1x PBS.
 3. Centrifuge at 1,200 x g for 5 min and discard supernatant. Resuspend the cell pellet with lysis buffer and incubate on a rocker at 4 °C for 30 min.

1. The amount of lysis buffer depends on the number of cells (e.g., 100 μ L for 1×10^6 cells); make sure to prepare fresh lysis buffer each time. For 1 mL of lysis buffer preparation (leave on ice): use 975 μ L M-PER Mammalian lysate buffer, 15 μ L protease and phosphatase inhibitors, and 10 μ L 100 μ M PMSF inhibitors.
4. Recover the supernatant protein by cell lysate centrifugation at 13,000 $\times$ g at 4 $^{\circ}$ C for 15 min.
5. Determine the protein concentrations by using a BCA protein assay.
6. Add 4x Laemmli Sample Buffer to protein samples (at least 20 - 30 μ g of protein) and heat at 100 $^{\circ}$ C for 3 min.
7. Use precast 4 - 20% gels and load 20 - 30 μ g of Laemmli protein solution and 5 - 10 μ L of protein standard per sample.
8. Fill the Western blot ring with TRIS/GLICINE /SDS 1x buffer and run gel at 110 V for 30 - 60 min (when time stops check that the dye has reached the end of the gel, otherwise run for a few additional minutes)
9. Electroblot to transfer protein into a PVDF membrane (see **Table of Materials**) by using a semi-dry system.
10. Block the membrane by soaking in the appropriate protein solution (milk/BSA 5%) on the shaker at room temperature for 1 h.
11. Make a 1:400 solution with anti-IQGAP1 primary antibody in a 5% milk solution (see **Table of Materials**).
12. Place the membrane in primary antibody solution on a shaker in a cold room overnight.
13. The following day, discard the antibody solution and soak in T-TBS 1x solution (1x T-TBS: 1x Tris Saline Buffer added by Tween20 0.1%) on a shaker at room temperature for 7 min.
14. Discard T-TBS 1x solution and replace. Repeat 3 times.
15. Make a 1:10,000 secondary mouse-antibody solution by adding antibody for standard Western blot detection (see **Table of Materials**) and place the membrane on a shaker; leave at room temperature for 2 h.
16. Discard the antibody solution and soak in T-TBS 1x solution on a shaker at room temperature for 7 min.
17. Discard T-TBS 1x solution and replace. Repeat 3 times.
18. Soak membrane for 5 min in a chemiluminescent solution, and image the membrane on a chemiluminescence imager.

3. Restoring Target-therapy Inhibitor Sensitivity by Candidate R-gene Knock-down

1. Cell preparation

1. Prepare single cell suspension by trypsinization. Perform transfection on the day that the cells are plated.
 1. Wash NCI-N87 cells with PBS and incubate at 37 $^{\circ}$ C with 0.05% trypsin/EDTA solution for 5 - 10 min. Add 2 volume of RPMI medium containing 10% fetal bovine serum to neutralize trypsin (e.g., 2 mL of medium for each 1 mL of trypsin).
 2. Count cells with a hemocytometer using trypan blue staining. Seed 2.5×10^5 cells (60% confluence) onto a T25 cm² flask for each condition.
NOTE: The appropriate number of cells for seeding is determined by the doubling time of the cell line and the duration of the experiment. The aim is to achieve the target gene knock-down for the entire duration of the experiment. Nine T25 cm² flasks are ideal for transfection conditions and the clonogenic assay (3 flasks for controls, 3 flasks for negative transfection controls, 3 flasks for gene-target transfection).

2. Reverse transfection

1. Prepare gene-target and negative control transfection mix for each T25 cm² flask as follows.
 1. Dilute 12.5 μ L of each gene-targeting siRNA oligonucleotides or Negative Control siRNA oligonucleotides in minimal essential transfection medium (ratio 1:55; see **Table of Materials**).
 2. Add a cationic liposome transfection reagent (1:1 ratio with gene-targeting siRNA oligonucleotides; see **Table of Materials**). Incubate at room temperature for 20 min.
NOTE: The total volume of the transfection mix is 700 μ L.
 3. Add 700 μ L of the transfection mix to each T25 cm² flask. Incubate cells at 37 $^{\circ}$ C for 1 - 3 days.
2. Verify gene knock-down by RT-PCR and Western blot analysis. Double-check the impact of the putative R-gene knock-down on the sensitivity restoration to the targeted therapeutic agent through colony formation experiments (repeat Step 1.5).

Representative Results

Figure 1 outlines the framework of the experimental procedure. Three gastric cancer cell-lines expressing a high level of HER2 and sensitive to trastuzumab (IC_{50} < peak plasma level of drug, **Figure 2A**, left graph) were grown in culture medium containing the targeted therapeutic agent. A dose 10-fold lower than the peak plasma concentration of trastuzumab (10 μ g/mL) was set as the starting dose, and gradually increased to 400 μ g/mL over a period of 8 - 12 months. Afterwards, we obtained three subclones characterized by a stable resistance phenotype with IC_{50} values lower than the peak plasma levels of trastuzumab (**Figure 2A**, right graph). In particular, the most resistant subclone, HR NCI N87, reached a RR IC_{50} value of 28.57 (**Figure 2B**). IQGAP1 appears to play a pivotal role in the resistance phenotype of the HR NCI N87 sub-line. The analyses of protein and gene expressions supported this hypothesis: the IQGAP1 protein level in the resistant subclone is about 5-fold higher than in the parental cells. Similarly, the correspondent IQGAP1 gene expression is 2.5-fold higher in the HR NCI N87 sub-line than in the NCI N87 line (**Figure 3A**).

IQGAP1 gene silencing was performed to evaluate its role in the development of the trastuzumab resistant phenotype. The protocol used to silence IQGAP1 gene expression proved particularly efficacious to knock down the IQGAP1 protein content on almost all of the cells grown in a T25 cm² cell culture flask (about 2.5×10^5 cells) (**Figure 3A**).

Furthermore, the clonogenic assay confirmed the working hypothesis, showing that IQGAP1 gene silencing restores trastuzumab sensitivity in HR NCI N87 cells as evidenced by the lowering of IC_{50} value to 7 μ g/mL (**Figure 3B**).

Figure 1: Framework for the *in vitro* analysis of the mechanisms underlying acquired targeted therapy resistance. Three different trastuzumab-resistant subclones derived from three different trastuzumab-sensitive cancer cell lines were generated and characterized for alterations in HER2-signaling mechanisms by next-generation sequencing, immunohistochemical, Western Blot, and RT-PCR techniques. All subclones showed a different resistance mechanism. Targeted-therapy sensitivity was restored in a specific subclone when candidate driver gene of resistance was silenced. [Please click here to view a larger version of this figure.](#)

Figure 2: Determination of relative resistance IC₅₀ index (RR IC₅₀) via clonogenic assay in gastric cancer cell lines. (A) Growth curves data for naïve (left graph) and resistant (right graph) cell-lines generated by colony forming experiments. The x-axis represents trastuzumab concentration. Error bars represent standard deviation (SD). The standard deviation never exceeded 5%. **(B)** Representative images of the clonogenic assay supporting the generation of a NCI-N87-resistant subclone. As shown by the time line, the generation of HR trastuzumab NCI N87 cells required 12 months of continuous exposure to the targeted agent. This figure is adapted from the work of Arienti *et al.*⁹ [Please click here to view a larger version of this figure.](#)

Figure 3: Restoring trastuzumab sensitivity by IQGAP1 gene knock-down. (A) IQGAP1 protein content, by densitometric analysis shown in the bar graph, in parental NCI N87 line, and in its resistant and silenced-resistant subclone (left graph). The mRNA IQGAP1 expression level was also verified in all the three cell-lines by RT-PCR technique (right graph). Data were presented as mean $\pm$ SD. (B) Clonogenic assay showed the different sensitivity of parental NCI N87 line and in its resistant and silenced-resistant subclone to 100 μ g/mL trastuzumab. The colony was counted after 14 days of cell seed. Thumbnail figures show representative colony cultures. This figure is adapted from the work of Arienti *et al.*⁹ Please click here to view a larger version of this figure.

Steps	Protocol	T25 cm ²
Seed cells 60% confluent	Adherent cells	1.75 x 10 ³
Dilute siRNA oligonucleotides in Minimal Essential Transfection medium (ratio 1:55)	siRNA	12.5 μ L
	Transfection medium	687.5 μ L
Add Lipofectamine transfection reagent	Lipofectamine reagent	12.5 μ L
Incubate	Incubate at room temperature for 20 min	
Add mixture to cells	Incubate cells at 37 °C for 1-3 d	

Table 1: Recommended reagent amounts and volumes for candidate resistance gene knock-down.

Discussion

While personalized and targeted therapies have elicited growing enthusiasm, these so-called 'smart' therapies face the same major hurdle as traditional chemotherapy drugs, *i.e.*, the rapid and unpredictable acquisition of drug resistance. To improve the results of targeted therapy, drug-resistance problems must be solved through a better understanding of the mechanisms that cause them. Here, we presented a widely accessible *in vitro* methodology to investigate the mechanisms of acquired resistance to targeted therapy drugs in cancer cell line models.

The generation of targeted therapy resistant subclones is the most critical and difficult to standardize step in this protocol, due to the intrinsic genetic heterogeneity between the cell lines used, and to their different degree of drug sensitivity. For these reasons, the time needed to obtain resistant subclones may vary. Chronic exposure to anticancer molecular agents typically causes a strong tumor growth inhibition. However, after a variable period of continuous drug exposure (8 - 12 months), cells resume growth with a rate similar to that of untreated control cells.

In addition, despite that gene expression data have been used to hypothesize gene-related target therapy resistance, a different expression level does not always reflect an improper protein function or correlate with the resistance phenotype. Owing to the complexity of the relationship between the expression of the putative drug-resistant gene and the resistance to the targeted therapy, further molecular investigations are needed, such as bioinformatics analysis, including gene/protein interaction, biological process enrichment, and molecular modeling.

Another critical point could be the specificity and the accuracy of siRNA oligonucleotide to reduce target expression. One possible solution could be the use of design tools for selecting functional siRNA, and a mixture of siRNA for the same target gene.

Finally, a minor limitation is related to the method used to restore the sensitivity to the targeted therapy inhibitor due to the transient duration of transfection. Maximal knock-down of target mRNA (>85%) was reported on day 2 post-transfection and was maintained >80% through days 5 - 7. Significant knock-down, albeit lesser in extent, was still observed on day 6. For this reason, the cytotoxicity assay for verifying the restoration of drug sensitivity should be performed within about 5 days from gene silencing.

Our protocol is a time- and cost-effective method for investigating *in vitro* acquired resistance. The generation of resistant subclones, mirroring the intra-tumor heterogeneity of cancer cells, could help unveil novel mechanisms of acquired resistance to anticancer targeted agents. In

particular, they are more suitable for high-throughput genomic or proteomic screenings for identifying molecular alterations in resistant cells than their sensitive counterparts.

This preclinical platform could be easily extended to investigate general resistance mechanisms to targeted therapeutic agents in different tumor histotypes, providing a standard procedure for the discovery of further drug targets to overcome drug resistance.

Disclosures

The authors have nothing to disclose.

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