

RNA Disruption and Drug Response in Breast Cancer Primary Systemic Therapy

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Abstract

Background: As there is now evidence that switching clinical nonresponders early in primary systemic therapy to alternate treatment regimens can enhance survival in some breast cancer patients, the need for a robust intermediate endpoint that can guide treatment response across all tumor subtypes is urgent. Recently, chemotherapy drugs have been shown to induce a decrease in RNA quality in tumor cells from breast cancer biopsies in some patients at midtherapy, and that this has been associated with subsequent achievement of pathological complete response (pCR). The decrease in RNA quality has been shown to be associated with RNA disruption; aberrant RNA bands visualized by RNA electrophoresis have been associated with subsequent tumor cell death. The objectives of these studies are to show that a new assay based on induction of RNA disruption in tumor cells by chemotherapy can stratify at midtherapy, pCR responders from non-pCR responders irrespective of clinical response and to present early evidence that clinically useful RNA disruption can be detected as early as 14 days after initiation of treatment.

Methods: RNA disruption in tumor cells was quantified by analysis of the RNA electrophoresis banding pattern and expressed as an RNA disruption index (RDI). To develop the RNA disruption assay (RDA), RDI was correlated with clinical outcome (pCR) from the NCIC-CTG MA.22 breast cancer clinical trial (ClinicalTrials.gov NCT00066443). RDA Zones were established by stratifying patients using RDI values into Zone 1, Zone 2, and Zone 3. Zone 3 included seven out of eight pCR responders, whereas Zone 1 contained no pCR responders. An intermediate zone (Zone 2) was established which contained one pCR. Subsequently, to determine early drug response, RNA disruption was examined by RDI after 14 days exposure to trastuzumab, zoledronic acid, or letrozole + cyclophosphamide ± sorafenib therapy.

Results: In MA.22, RDA stratified 23 of 85 patients in Zone 1 as pCR nonresponders, 24 patients in Zone 2, an intermediate zone, and 38 patients in Zone 3, pCR responders and non-pCR patients who share RDI comparable to those achieving pCR. In the early response studies, after 14 days exposure to chemotherapy, some RNA disruption as measured by RDI elevation could be detected in 3/12 trastuzumab, 7/15 zoledronic acid, 5/29 letrozole + cyclophosphamide, and 5/23 letrozole + cyclophosphamide + sorafenib patients.

Conclusions: RDA is a novel intermediate endpoint that has promise for clinical utility for breast cancers early in response-guided primary systemic therapy.

It is well recognized that primary systemic therapy results in an increase in disease-free survival (DFS) only in a minority of breast cancer patients (1,2), even though most tumors shrink with

treatment as assessed by palpation and imaging techniques (3). Because all chemotherapy patients experience severe side effects whether or not treatment has an enhanced survival benefit, the

search for an effective intermediate endpoint for drug response in primary systemic therapy of breast cancer has been a long sought goal (4–6). As switching treatment of nonresponders early in therapy to an alternate regimen can enhance survival (7), the need for a test to identify chemotherapy nonresponders across tumor subtypes early in chemotherapy is increasingly urgent (8).

An ideal intermediate endpoint would be able to stratify patients for future pathological complete response (pCR) and DFS sufficiently early in therapy to be useful for decision making. Where the endpoint indicates that the patient is not responding or is inadequately responding to therapy, modification of therapy could be considered. Where the endpoint indicates that the patient is responding to therapy, reassurance can be provided for both patients and their physicians. Validating such an endpoint using archived samples by strict level of evidence criteria is recognized as a formidable task (9). These criteria include: adequate archived samples from a completed prospective clinical trial, preanalytical and analytical reproducibility, focus on a single classifier, and clinical validation through one or more similar studies involving independent populations (9).

Discovery of RNA Disruption and RNA Disruption Assay Development

While performing routine tumor RNA quality assessments by capillary electrophoresis on the Agilent 2100 Bioanalyzer for the MA.22 neoadjuvant breast cancer clinical trial, Parissenti et al. (10), Laurentian University, Sudbury, Canada observed that patients achieving pCR uniformly had decreased RNA quality as expressed by RNA integrity number in tumor samples obtained at midtreatment (three or four chemotherapy cycles).

Review of the midtreatment RNA electrophoresis patterns in those patients who proceeded to pCR revealed multiple aberrant bands mainly between the 28S and 18S regions as well as reduced 28S and 18S peaks. We termed these abnormal bands collectively as “RNA disruption.” Less intense RNA disruption bands were present as well in some patients who did not achieve pCR. RNA disruption bands were generally absent in pretherapy samples and were distinct from fast region peaks associated with RNA autolytic degradation (11) RNA cleavage peaks described with apoptosis (12,13) may overlap and contribute to a component of the RNA disruption pattern.

In cell culture, chemotherapy drugs are known to alter ribosomal RNA (rRNA) metabolism (14) and ribosome biogenesis at levels of rRNA transcription as well as early or late rRNA processing (15). Fimognari et al. (16) detected RNA damage as aberrant patterns in RNA electropherograms from cell cultures exposed to doxorubicin and other agents. Copois et al. (17) observed compromised RNA quality likely associated with ischemia in human colon cancer tissues in vivo. About 85% of cellular RNA is within the ribosome. Each ribosome contains four RNA molecules, 5S, 5.8S, 18S, and 28S, collectively termed rRNA. rRNA is visualized as the only distinct peaks in normal RNA electrophoresis. rRNA is usually very highly conserved although rRNA disorders known as ribosomopathies are now recognized (18). Intact rRNA is necessary for full ribosome function and cell survival. If RNA disruption affects only a portion of cells or if RNA disruption is subcritically present in all tumor cells, it is likely that the cells can survive with compromised function. When rRNA structure is sufficiently disrupted, the cells may no longer be able to make proteins and other substances necessary for cell division. If rRNA disruption is more extensive, the cells would not be able to maintain their basic functions and the cells die. Accordingly, we set out to develop a RNA disruption assay (RDA) which could

identify and quantify rRNA disruption in the RNA electropherogram derived from tumor cells and to associate the degree of rRNA disruption with chemotherapy response.

The objectives of these studies are to show that the RDA for RNA disruption can stratify at midtherapy, pCR responders from nonresponders, and to present early evidence that clinically useful RNA disruption can be detected as early as 14 days after initiation of therapy.

Methods

MA.22 Trial

The MA.22 trial (ClinicalTrials.gov NCT00066443) compared drug efficacy of various combination regimens of epirubicin and docetaxel in primary systemic therapy for locally advanced breast cancer. Hormone responsive (HR+ve), HR+ve HER2+ve, HR-ve HER2+, and triple-negative breast cancer subtypes, were all represented (10). Epirubicin and docetaxel were administered to patients with locally advanced breast cancer in either a standard (q three weekly) or dose dense (q two weekly) regimen, with pegfilgrastim support and biopsies taken pretherapy, midtherapy, and posttherapy as previously described (10).

For RNA isolation, the frozen biopsies were placed into 0.5 mL of RLT buffer (component of the Qiagen RNeasy Mini RNA isolation kit), and processed as previously described (10).

RNA electrophoresis was performed on the Agilent 2100 Bioanalyzer using same methods as that to obtain RNA integrity number to assess RNA quality (11). The raw data from each electropherogram was exported and subsequently used for RDI assessment by proprietary algorithms.

RDA Development

The RDA is an assay to assess quantitatively, RNA disruption present in tumor cells and to associate the response to therapy to the amount of disrupted RNA relative to normal RNA in tumor cells. To develop the RDA, using tumor RNA electropherogram data from the MA.22 trial, we developed proprietary methods to quantify the proportion of disrupted RNA to normal RNA in each sample and to express the disrupted RNA/normal RNA as a RNA disruption index (RDI). Subsequently, RDI data from 85 MA.22 patients who completed the trial were stratified into three RDA zones based on their midtreatment RDI and subsequent pCR response. pCR was defined strictly as no residual tumor in either the breast or axillary regional nodes. We established a priori, a Zone 1, Zone 2 cutoff with negative predictive value for pCR greater than 0.99 (RDI = 10). Below this cutoff level, the chance of achieving pCR is less than 1%. Zone 3 was established above the Zone 2, 3 cutoff level with a positive predictive value for pCR of greater than 2 (RDI = 35).

Cremona: Window-of-Opportunity Trials

Core biopsies were obtained at baseline and 14 days after start of therapy. The biopsies were placed in RNA later and subsequently fast frozen. Samples were then thawed and shipped with ice packs to Dr. Parissenti's lab in Sudbury, Ontario, Canada. RNA was then isolated as described above (10). Preliminary data was obtained in biological window-of-opportunity trials in three trials, zoledronic acid, trastuzumab, letrozole + cyclophosphamide and letrozole + cyclophosphamide + sorafenib in breast cancer T2-4, N 0–1, M0 after 14–21 days exposure, with control biopsies pretherapy, Table 1, and Table 2. The limit of detection for RDI was previously established as RDI = 0.3. RDI = 1.5, five times

the level of detection, was used as a provisional baseline cutoff between nonresponders and responders.

Results

MA.22 Studies

Figure 1 demonstrates the distribution of MA.22 patients in the RDA zones. RDI is plotted on the vertical axis, RNA concentration on the horizontal axis, both on the log scale. Zone 3, the responder zone incorporated seven of eight pCRs and Zone 2 incorporated one pCR; Zone 1 by design, the nonresponder zone, had no pCRs. RDA showed 23 patients (27%) distributed in Zone 1, 24 patients, Zone 2 (28%), and 38 patients in the responder Zone 3 (45%) As well as seven pCRs, Zone 3 had 21 HR+ve, three HER2, and seven triple-negative breast cancer patients who did not obtain pCR. RDI can be measured accurately over three orders of RNA concentration magnitude 10–1000 ng/μL with

limit of detection less than 10 ng/μL. HR+ve, HER2+ve, and triple-negative breast cancer subtypes were present in all three zones. Specifically, midtreatment RDI did not correlate with tumor shrinkage as observed clinically during therapy.

Cremona: Window-of-Opportunity Trials

For a response intermediate endpoint to be maximally effective, it is desirable that the endpoint demonstrate effect early in chemotherapy, preferably within the first 2 weeks of starting therapy. Table 1 summarizes the regimens for the window-of-opportunity studies, the responding patients by RDI criteria, the RDI values associated with patients who had clinical progression and the pCR response. Table 2 summarizes the chemotherapy regimens of these patients before surgery.

Most striking, by the provisional cutoff criteria for response of five times or higher elevation of RDI from baseline, eight of 15

Table 1. RNA disruption, 14–21 days after initiation of therapy*

Drug	Patients total	Patients responding†	RDI range, responding patients	Progressing patients and RDI
Trastuzumab (dose: 6 mg/kg)	12	4	For 3 patients 1.6–2.3 For one patient pCR RDI= 1.4	2 RDI = 1.1 RDI = n/a
Zoledronic acid (dose: 4 mg)	15	7	1.5–8.9	1 RDI = 0.8
Letrozole (dose: 2.5 mg/d) + cyclophosphamide (50 mg/d) metronomic dosage	29	5	1.7–3.5	2 RDI = 0.4 RDI = 0.6
Letrozole (dose: 2.5 mg/d) + cyclophosphamide (dose: 50 mg/d) + sorafenib (dose: 400 mg bid) metronomic dosage	23	3	2.0–3.3	1 RDI = n/a

*n/a = not available; pCR = pathological complete response; RDI = RNA disruption index.

†Patients with RDI above baseline RDI = 1.5. RDI = 1.5 is five times RDI, level of detection, RDI = 0.3, RDI version 7.

Table 2. Clinical trials*

Clinical trial	Chemotherapy	Patients	Biopsy samples
MA.22	Epirubicin, docetaxel combination therapy: q 3 weekly regimen epirubicin 75 mg/m ² ; docetaxel 75, 90, 105, or 120 mg, q 2 weekly regimen 50, 60, and 70 mg/m ² For both drugs, 6–8 cycles, surgery	Locally advanced breast cancer, HR+ve, HER2+, TNBC 96 enrolled, 85 completed, receptor data = 82	•Before therapy •At 3–4 cycles •Posttherapy
ZOLD	4 mg IV day 1 After biopsy, epirubicin 40 mg/m ² /w/ q21 + cyclophosphamide 600 mg/m ² 1–21 4 cycles, surgery	Locally advanced breast cancer 33 enrolled, 29 completed	•Before therapy •Day 14
TRUP	8 mg/kg, IV day 1 After biopsy, docetaxel 100 mg/m ² 1–21 + trastuzumab 6 mg/kg 1–21 4 cycles, surgery	HER2+ve 12 patients enrolled	•Before therapy •Day 21
Letrozole, cyclophosphamide ± sorafenib	Letrozole 2.5 mg/d + cyclophosphamide 50 mg/d + Sorafenib 400 mg bid metro- nomic dosage for 6 mo Surgery	Postmenopause T2–4, N0–1 M0 ER+, HER2– 2923	•Before therapy •Day 14

*ER+ = estrogen receptor responsive; HR+ve = hormone responsive; HER2+ve = HER2-overexpressing; TNBC = triple-negative breast cancer.

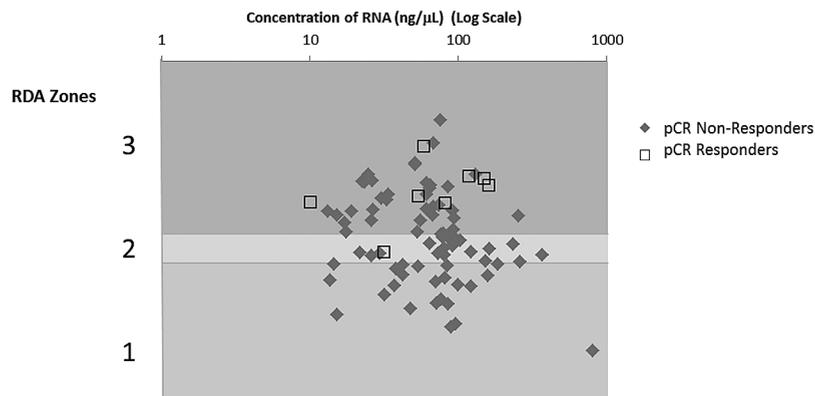


Figure 1. Ma-22 patients: RNA disruption assay (RDA) Zone distribution. pCR = pathological complete response.

patients exposed to zoledronic acid; five in the letrozole-cyclophosphamide group, five in the letrozole + cyclophosphamide + sorafenib, and three in the trastuzumab group showed elevated RDI. One trastuzumab patient achieved a pCR with a borderline RDI = 1.4. RDI did not correlate with tumor response as observed by clinical positron emission tomography or magnetic resonance imaging in any of the studies. Six progressors have been identified to date with RDI values ranging from RDI = 0.4 to 1.1.

Discussion

Primary systemic therapy has facilitated the study of intermediate endpoints which are candidates to have clinical utility as prognostic biomarkers for future responses including pCR, clinical progression and ultimately, DFS (4). Candidate prognostic markers include clinical response, immunohistochemical markers such as Ki-67, imaging including magnetic resonance imaging, positron emission tomography, and ultrasound but to date none have had sufficiently high correlation with either pCR response or clinical progression to be widely useful in therapeutic decision making (3,4,19,20). Similarly, molecular tests before therapy usually apply to a limited set of drugs such as anthracyclines and cyclophosphamide and while generally predictive for nonresponse in the population also show that individuals receiving chemotherapy have higher survivability than those who do not (21).

Primary systemic therapy has enabled the introduction of response-guided therapy whereby the chemotherapy can be changed for those patients who are clinical nonresponders (7). Clinical response, a standard prognostic criterion is considered a much weaker intermediate marker than pCR (22,23). Nonetheless, von Minckwitz et al. (7) demonstrated in a controlled trial, enhanced survival for patients lacking clinical response after two chemotherapy cycles who subsequently were placed on alternate therapy. Paradoxically, enhanced survival was greatest in HR+ve patients and could not be predicted by pCR. pCR is recognized as the best current intermediate endpoint of chemotherapy to predict DFS (24). For an individual patient, achieving pCR within their breast cancer can indicate enhanced survival (25). However, in a meta-analysis this prediction did not apply at the clinical trial level reflecting that other factors may contribute to enhanced survival (25). Further, as less than 10% of HR+ tumors achieve pCR and as pCR is available only after treatment, pCR's utility for response-guided chemotherapy is severely limited (1,26,27). Accordingly there is a great need for a biomarker superior to pCR that can provide prognostic information for response-guided therapy. Like pCR, RDI is a direct marker of drug effect to incapacitate tumor cells. Unlike pCR, as a clinical test, RDI is incorporated into RDA and can be assessed early in therapy before cell death is complete

within the tumor. For Zone 3 patients, RDA may have predictive power for chemotherapy response not only for patients achieving pCR but for the entire Zone 3 tumor population irrespective of subtypes. This suggests that RDA in Zone 3 may be assessing extensive cytostatic effects of chemotherapy as well as cell death. RDA Zone 2 includes those patients responding insufficiently to therapy and who may benefit from extended or alternate treatment. Most important, in Zone 1, RDA predicts strongly those patients who are not responding to chemotherapy and are extremely unlikely to achieve a pCR.

These results raise the question whether measuring enhanced survival by pCR particularly in HR+ patients, underestimates considerably the effects of current drugs on DFS.

While the early response studies are preliminary, the results indicate that in some tumors, RDI elevation can be detected as early as 2 weeks after start of metronomic therapy. Further, for each of the drug regimens studied, our data suggest that RNA disruption takes place in response to a variety of structurally distinct chemotherapy agents, not just anthracyclines and taxanes as previously demonstrated in the MA.22 study. Moreover, our data suggests that differences between responders and nonresponders can be detected by RDI as early as day 14 after chemotherapy initiation. Future research to validate RDA, which is in progress, includes the study of independent chemotherapy data sets for which RNA disruption data and clinical outcomes are available, further examination of response stratification with additional datasets early in chemotherapy, and experimental studies of RNA disruption and its associations with mechanisms of subsequent cell impairment and eventual cell death.

Conclusions

The search for an effective intermediate endpoint useful for guiding therapeutic response has encompassed imaging, molecular, and histochemical markers but up to the present, none has proved more accurate than pCR (27). RDA can stratify well the portion of the patient population that will and will not subsequently develop pCR but this remains to be validated in an independent patient population. These studies are in progress. RNA disruption can be assessed early during therapy at a time when treatment changes can still be considered for RDA nonresponders. Elevated RDI can be detected in some tumors as early as 14 days after initiation of chemotherapy raising the prospect of assessing chemotherapy response by window-of-opportunity trial in individual patients. Presently, RDA appears to be a most promising prognostic intermediate endpoint for use in response-guided primary systemic therapy for breast cancer.

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