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CLINICAL TRIAL

Association of low tumor RNA integrity with response to chemotherapy in breast cancer patients

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Abstract The CAN-NCIC-MA22 phase I/II clinical trial evaluated women with locally advanced or inflammatory breast cancer treated with epirubicin and docetaxel at 2 or 3 weekly intervals in sequential cohorts. The relationship between various biomarkers and treatment response was assessed. Breast biopsy cores were obtained from 50 patients pre-, mid-, and post-treatment. Immunohistochemical staining was performed to determine baseline levels of estrogen receptor (ER), progesterone receptor (PR), Her2/Neu protein (HER2), and topoisomerase II (Topo 2), expressed as percent positive stain. Tumor RNA integrity (RIN) and tumor cellularity were measured pre-, mid- and post-treatment by capillary electrophoresis and light microscopy after hematoxylin/eosin staining, respectively. Associations between 1) maximum RIN and 2) tumor cellularity at the three time points with baseline levels of ER, PR, Her2, and topo II were assessed using Spearman and Pearson correlation coefficients. Associations between RIN and tumor cellularity with chemotherapy dose level or pathologic response were assessed using one-way ANOVA. In this study, we observed that low mid-treatment maximum RIN (but not tumor cellularity) was associated with high chemotherapy drug dose level ($P = 0.05$) and eventual pathologic complete response (pCR) ($P = 0.01$). Post-treatment, low maximum RIN was found to be associated with low tumor cellularity ($P = 0.004$), and low tumor cellularity with pCR ($P = 0.01$). Post-treatment tumor cellularity was lowest in patients with tumors having high baseline PR levels ($P = 0.05$). The association of mid-treatment RIN with drug dose level and with pCR suggests that tumor RIN may represent an important new biomarker for measuring response to chemotherapy in breast cancer patients.

Keywords Docetaxel · Epirubicin · Breast neoplasms · Cancer treatment · Clinical trial, phase I · Clinical trial, phase II · Biomarker · Drug response · RNA integrity

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Introduction

Among the most effective systemic chemotherapy agents in breast cancer management are the anthracyclines (typically doxorubicin or epirubicin) and taxanes (paclitaxel or docetaxel). Anthracyclines disrupt the uncoiling of DNA by topoisomerase II α (Topo 2) [1], intercalate between DNA strands [2], and cause DNA lesions [3]. Taxanes block the depolymerization of microtubules [4], resulting in mitotic arrest and apoptosis [5, 6]. Despite their effectiveness, patient tumors can exhibit intrinsic or acquired resistance to these agents [7, 8]. A variety of mechanisms associated with resistance to anthracyclines and taxanes have been identified [9–20].

Unfortunately, there is currently no definitive approach to distinguish between drug-responsive and drug-resistant

tumors. Monitoring the chemotherapy-induced reduction in uptake of ^{18}F-deoxyglucose (FDG) in breast tumors by positron emission tomography (PET) has shown significant promise in measuring response to chemotherapy mid- or post-treatment [21–23]. However, the sensitivity of this approach is only 23% in common lesions that are less than 10 mm [24] and in well-differentiated or slowly growing tumors that have lower rates of glucose metabolism [25, 26]. In addition, there is considerable overlap between the magnitude of reduction in FDG uptake between responding and non-responding tumors [22]. Thus, it would appear that alternative or additional methods may be required to accurately measure tumor response to chemotherapy in patients with breast and other cancers.

Chemotherapy agents may induce sufficient damage to tumor cells (directly or through effects on rapidly dividing endothelial cells associated with the tumor vasculature [27]) to result in dramatic reductions in tumor RNA quality through the release of lysosomal RNAses. If so, then reductions in tumor RNA quality during or post-treatment may serve as a useful biomarker of patient response to chemotherapy. RNA quality can be measured by the ratio of the 28S and 18S rRNAs in tumors or by the tumor RNA integrity number (RIN). In the latter case, a RIN of 10 represents high quality, intact RNA, while 0 represents completely hydrolyzed RNA. The RIN has been shown to be superior to the 28S/18S rRNA ratio for reliably measuring RNA quality [28–30] and is emerging as the "gold standard" for RNA quality assessment in mammalian cell lines and tissues [31], including breast tumors [30].

In this study, we examined RNA integrity and other biomarkers in tumor biopsies of locally advanced breast cancer patients before, during, and after epirubicin/docetaxel chemotherapy in association with a clinical trial (MA22) by the National Cancer Institute of Canada Clinical Trials Group (NCIC CTG). We observed that tumor RIN values (unlike tumor cellularity) were significantly reduced in these patients in response to epirubicin/docetaxel chemotherapy mid-treatment and that low mid-treatment tumor RIN values correlated with pathological complete response (pCR) post-treatment.

Methods

MA22 Goals, design, and patient inclusion criteria

CAN-NCIC-MA22 (ClinicalTrials.gov identifier NCT00066443) is a phase I/phase II clinical trial, whose goal is to determine optimum dosing regimens for docetaxel/epirubicin combination chemotherapy in women with locally advanced or inflammatory breast cancer. Patients are treated with various doses of epirubicin and docetaxel (with pegfilgrastim support) using a standard (q21) or dose dense (q14) regimen. The primary goal of the phase I portion of the trial (now completed) was to determine the maximum tolerated dose (MTD) for patients under each dosing regimen, while the phase II portion is assessing response rates and toxicities at the MTD for both regimens. Doses for the q21 regimen were 75 mg/m^2 IV of docetaxel and 75, 90, 105, or 120 mg/m^2 IV of epirubicin (with 6 mg pegfilgrastim per cycle on day 2). Doses for both docetaxel and epirubicin in the q14 regimen are 50, 60, and 70 mg/m^2 IV (with identical pegfilgrastim support). In the phase II portions of the trial, response to chemotherapy at both dosing schedules is being assessed by determining the clinical and pathological response rates and the duration of response at the recommended phase II doses.

Only women with no prior cytotoxic therapy and no evidence of metastatic disease are permitted to enroll in the trial. Patients are allocated to the various phases and dosing regimens of the trial in a non-randomized fashion. In phase I, only a single patient was allocated at a time during dose escalation, in order to identify dose-limiting toxicities and to identify the maximally tolerated dose for the q21 and q14 regimens. Accrual has been completed for phase I of both regimens and for phase II of the q21 regimen. Phase II accrual continues for the q14 regimen. The secondary goal of the trial is to identify potential biomarkers of response to epirubicin/docetaxel chemotherapy, one of which is the subject of this study. Written informed consent was obtained from each subject. The trial is being administered through the NCIC Clinical Trials Group (NCIC CTG), who assigns patients to the various phases and dosing regimens. It was approved by Health Canada and activated on Feb 25, 2003 at various sites within Canada (predominantly the Odette Cancer Centre and Princess Margaret Hospital in Toronto), only after obtaining approval by each institution's research ethics committee. Phase I and Phase II of the q21 regimen closed to accrual on September 13, 2004 and May 10, 2006, respectively. Phase I of the q14 regimen closed on November 5, 2007, while the phase II portion of the q14 regimen is still accruing patients.

In terms of sample size considerations, fifteen response-evaluable patients were entered into the first stage of each phase II regimen. Since >7 responses were observed in the patients, accrual was permitted to stage II, where another 15 response-evaluable patients were accrued to each regimen to obtain 30 patients per regimen. The trial will test the null hypothesis that the response rate is 45% or less versus the alternative hypothesis that the response rate is greater than 70%. The significance level (i.e., the probability of rejecting the null when it is true) is 0.035, and the power (i.e., the probability of deciding the regimen is active) is 0.83 when the response rate is 70%. A 95%

confidence interval of the estimated response rate is being used as an indicator of the effectiveness of the regimen.

Tissue sampling by ultrasound-guided core biopsy

Tumor core biopsies were harvested from patients before, during, and after treatment in order to identify potential biomarkers of response to the regimens. The size and location of the lesion at each time point was documented using the linear array L12-5 transducer of the Philips ATL HDI 5000 SonoCT ultrasound system (Bothell, WA). The skin and breast tissue near the lesion were infiltrated with 1% lidocaine and a skin nick was made with a scalpel. Under ultrasound guidance, six core biopsies were obtained through the same incision using a 14-gauge needle mounted on an automated spring-loaded device. Three biopsies were fixed in formalin for embedding, sectioning, and immunohistochemical studies, while the remaining three biopsies were flash-frozen for RNA quality assessments.

Isolation of total RNA from breast tumor core biopsies and evaluation of RNA integrity

RNA was isolated from the above tumor core biopsies using Qiagen RNAeasy^{TM} Mini kits (Qiagen Laboratories, Mississauga, ON). After removal from cryostorage, the biopsies were immediately immersed in 0.5 ml of RLT buffer containing 1% β-ME and homogenized with a coreless motor homogenizer (Kontes Glass Company) for 5 min. The lysate was passed 5 times through a 20-gauge needle fitted to a syringe, centrifuged at high speed in a microfuge for 3 min, and the RNA in the supernatant extracted and purified as per the manufacturer's protocol. RNA was eluted from Qiagen columns in 30 µl of RNase-free water. An aliquot of the eluted RNA was subjected to capillary electrophoresis on an Agilent 2,100 Bioanalyzer, and the quantity and integrity (RIN value) of the RNA determined using associated software. RIN values termed "n/a" (often due to very low yields of RNA from the biopsy) were assigned a value of "0".

Measurement of levels of tumor marker proteins and tumor cellularity

The levels of various tumor marker proteins were determined by standard immunohistochemistry using antibodies to ER (clone 6G11, Novacastra Labs), PR (Clone 16, Novacastra Labs), HER2 (TAB 250, Zymed Labs), and Topo 2 (clone SWT3D1, Dako Labs). Levels were expressed as percent positive stain. Tumor cellularity (extent) was determined by staining sections of core biopsies with hematoxylin/eosin and computing the percentage of cells in the section with tumor cell morphology (as determined by pathologist Dr. Harriette Kahn).

Statistical analysis of data

This report covers results for the first 50 patients, approximately half of the total patients who will be enrolled to CAN-NCIC-MA22. Baseline tumor characteristics (including ER, PR, HER2, and Topo 2) were determined immunohistochemically as percent positive stain, for which frequency histograms were produced. Formal testing for adequacy of assuming normality for RIN, ratio of 28S/18S, tumor cellularity, and each tumor marker was with Shapiro–Wilk, Kolmogorov–Smirnov, Cramer-von Mises, and Anderson–Darling test statistics. If the assumption of normality was inappropriate, a Box-Cox transformation of the data [32] was performed to improve symmetry and stabilize variances.

Associations between (transformed) 1) maximum RIN and 2) tumor cellularity at the three time points with baseline levels of ER, PR, HER2, and Topo 2 were assessed using Spearman and Pearson correlation coefficients. The associations between RIN and tumor extent with clinical T stage, dose level, clinical response, and pathologic response were assessed using one-way ANOVA; when there was evidence of heterogeneous variances by the Levene test ($P \leq 0.10$), a Welch test statistic was used. Additionally, we plotted maximum RIN at the three time points versus drug dose, and versus tumor cellularity.

Results

Baseline patient characteristics, the maximum tolerated doses, and common toxicities observed in the NCIC-MA-22 clinical trial

The CONSORT (Consolidated Standards of Reporting Trials) flow diagram for the CAN-NCIC-MA22 clinical trial is depicted in Fig. 1. Only patients assessed for tumor integrity, tumor cellularity, and other biomarkers of response to chemotherapy are depicted in the CONSORT diagram. Baseline patient and tumor characteristics for our study are indicated in Table 1. Accrual to the trial continues for the phase II portion of the q14 regimen. The maximum tolerated doses in NCIC-MA-22 patients receiving neoadjuvant docetaxel/epirubicin chemotherapy at the standard (q21) and dose dense (q14) regimens was found to be 105 mg/m^2 epirubicin/75 mg/m^2 docetaxel and 60 mg/m^2 epirubicin/60 mg/m^2 docetaxel, respectively. Common toxicities in the phase 1 trials were febrile neutropenia and diarrhea (in one patient) for the q21 regimen,

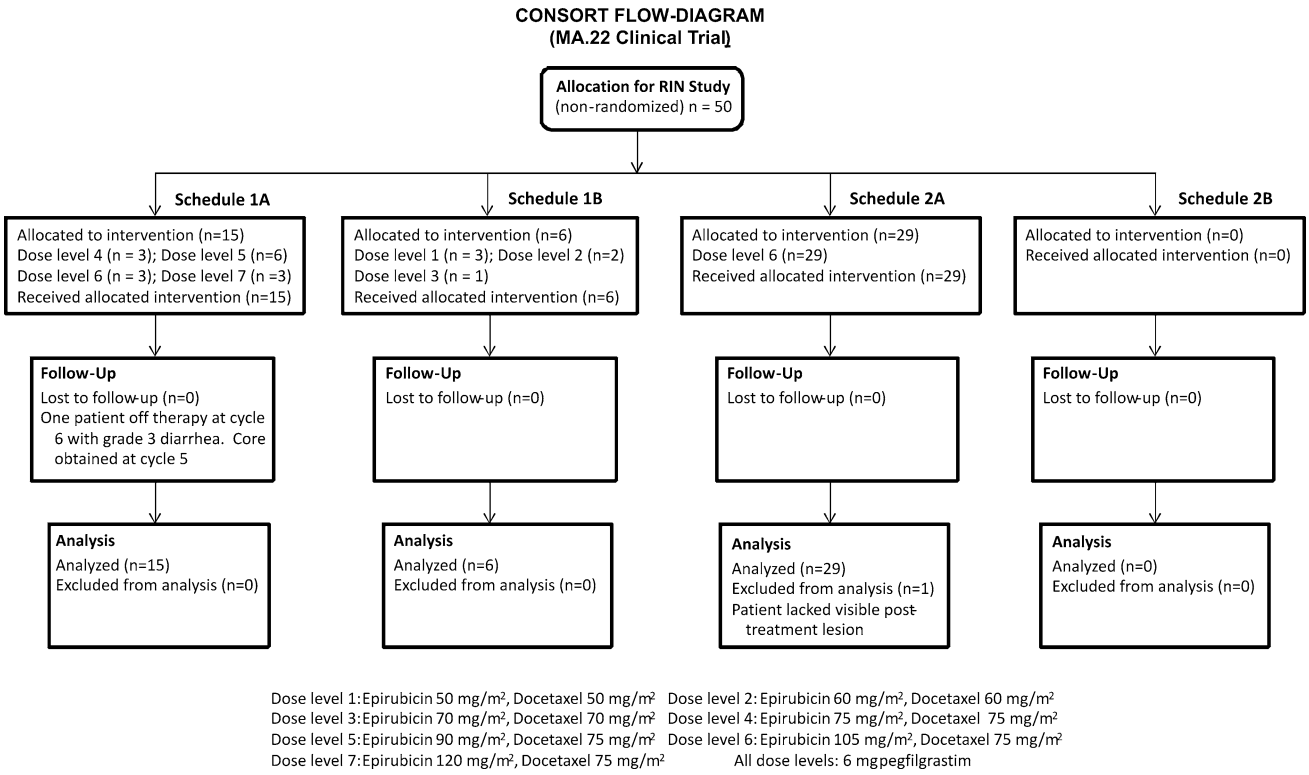

Fig. 1 CONSORT (Consolidated Standards of Reporting Trials) flow diagram for the CAN-NCIC-MA22 clinical trial

and fatigue (100%), alopecia (100%), nausea (83%), stomatitis (67%), and heartburn (67%) for the q14 regimen. Common toxicities in phase II of the q21 regimen were fatigue (97%), alopecia (94%), nausea (90%), stomatitis (61%), hot flashes (52%), diarrhea (48%), myalgia (45%), and anorexia (41%).

Relationship between tumor RIN values and RNA quality

RIN was assessable for all 50 patients pre- and mid-treatment. One post-treatment biopsy was not available for RIN assessment due to the lack of a visible lesion. Similar to previously reported findings [28–30], we observed that the RIN is a reliable measure of RNA integrity, as visualized by inspection of electropherograms of biopsy RNA preparations after capillary electrophoresis. The RIN value for a particular tumor RNA preparation was clearly proportional to the intensity of the 28S and 18S rRNA bands on electropherograms (Fig. 2). Consistent with the RIN as an effective measure of RNA quality, there was a very strong association pre-, mid-, and post-treatment between maximum tumor RIN values and 28S rRNA/18S rRNA ratios—an alternative measure of RNA quality (P values as low as < 0.0001). A Box-Cox transformation was required for RIN to improve data symmetry and stabilize variances (power 2).

Changes in RNA integrity and tumor cellularity mid- and post-treatment

Figure 3 represents a plot of the mean maximum tumor RIN value for each MA22 patient before, during, and after treatment. RIN values were not significantly different at baseline ($P = 0.45$), but strongly decreased in some patients mid- and post-treatment. Low tumor RIN values were associated with high drug dose level mid-treatment ($P = 0.05$) and post-treatment ($P = 0.12$). Mean maximum RIN values are indicated in Table 2 for patients at various dose levels. Pre-, mid-, and post-treatment mean maximum RIN values were, respectively, 6.5 (95% confidence interval, 6.1, 6.8), 3.8 (3.0, 4.5), and 4.2 (3.2, 4.5). Baseline RIN 95% confidence intervals exclude those for both mid- and post-treatment. Despite the small number of patients ($n = 3$), the effect of chemotherapy on tumor RIN values was particularly evident for tumors exposed to epirubicin/docetaxel at dose level 7, where the mean maximum tumor RIN values were 5.2 pre-treatment and 0.0 both mid- and post-treatment (Table 2).

Figure 4 (panel A) is a plot of maximum tumor RIN against tumor cellularity for MA22 patients before, during,

Table 1 Baseline patient and tumor characteristics [patient characteristics ($N = 50$): median age 47.0 years (range 27.4–72.3)]

Factor	Number of patients
ECOG performance status	
0	49
1	1
Measurable disease	
Yes	50
No	0
Histology/clinical	
Infiltrating ductal	41
Inflammatory	6
Lobular	3
N Clinical classification	
N1	17
N2	23
N3	4
NX	6
T Clinical classification	
T2	2
T3	17
T4	30
TX	1
Factor	**Median (range)**
Pre-treatment tumor characteristics	
ER (% of cells with positive staining)	45 (0–100)
PR (% of cells with positive staining)	1 (0–100)
HER2 (% of cells with positive staining)	0 (0–100)
Topo 2 (% of cells with positive staining)	70 (0–100)
Tumor cellularity (% tumor cells)	100 (40–100)
RIN	6.9 (0–8.7)

and after treatment. It shows a highly variable but general reduction in both RIN and tumor cellularity mid- and post-treatment when compared with baseline. Figure 4 (panel B) contains photomicrographs of tumor core biopsies with corresponding maximum RIN values for representative MA22 patients before, during, and after chemotherapy. As shown in Fig. 3 or Fig. 4 (panel B), patients exhibited 1) no change in RIN values in response to treatment, 2) a temporary reduction in RIN mid-treatment only, 3) a reduction in RIN post-treatment only, or 4) reductions in RNA quality mid- and post-treatment. For a small number of patients, tumor RIN was poor at all time points. In general (but not always), there was a good concordance between maximum RIN and tumor cellularity post-treatment (Fig. 4, panel B). *P* values for Pearson and Spearman correlations between maximum RIN and tumor cellularity post-treatment were 0.0003 and 0.004, respectively (Table 3).

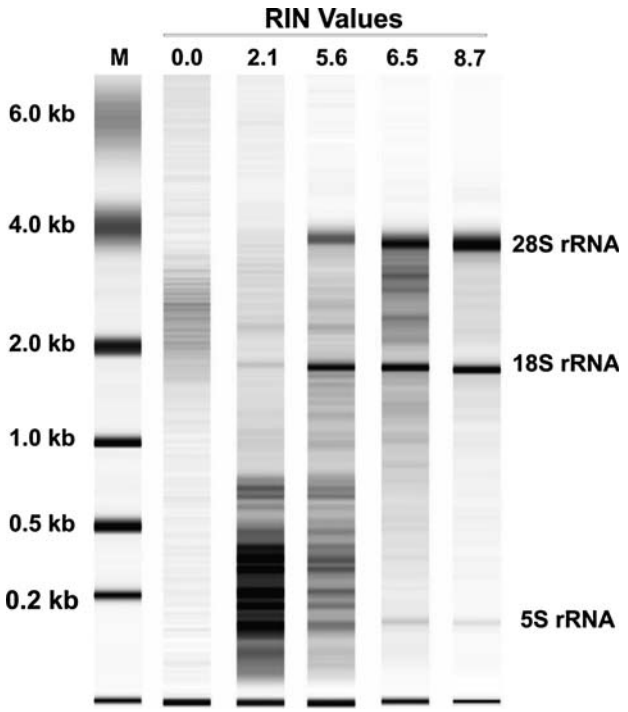

Fig. 2 Relationship between tumor RIN and tumor RNA quality. The tumor RNA integrity number (*RIN*) is listed for various MA22 tumors. The RNA quality of the corresponding tumor is also depicted, as measured by the appearance of bands on electropherograms of the tumor RNA preparations after capillary electrophoresis

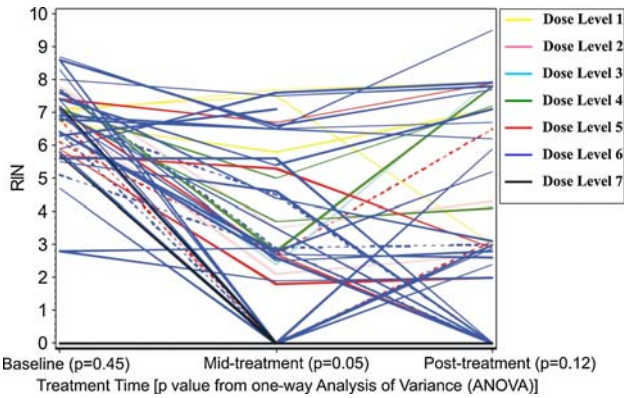

Fig. 3 Relationship between tumor RIN and drug dose level at various treatment times. Maximum tumor RNA integrity number (*RIN*) values for each patient are plotted by treatment period (pre-, mid-, and post-treatment) and drug dose: dose level 1 (*yellow*), 2 (*pink*), 3 (*cyan*), 4 (*green*), 5 (*red*), 6 (*blue*), and 7 (*black*). *P* values are for differences in RIN at time points. Patients exhibiting pathologic complete response are indicated with dashed lines

Relationship of tumor RNA integrity and tumor cellularity with response to chemotherapy

Of the 50 patients evaluated in this study, 13 patients were observed to have a clinical complete response (cCR), while 37 patients were deemed non-responders—defined as a

Table 2 Tumor RIN values by treatment time and dose level

Treatment time	Dose level[a]	*N*	Mean	95% Confidence interval
	All patients	50	6.5	(6.1, 6.8)
Baseline	1	3	6.9	(6.7, 7.2)
	2	2	N/A[c]	(6.4, 8.2)
	3	1	7.2[b]	(N/A, N/A)
	4	3	7.3	(6.8, 7.7)
	5	6	6.5	(5.9, 7.0)
	6	32	6.3	(5.8, 6.9)
	7	3	5.2	(0.0, 7.7)
	All patients	50	3.8	(3.4, 4.5)
Mid-treatment	1	3	7.0	(5.8, 8.1)
	2	2	N/A[c]	(1.0, 4.0)
	3	1	2.4[b]	(N/A, N/A)
	4	3	3.8	(2.2, 5.0)
	5	6	3.3	(0.9, 4.6)
	6	32	3.8	(2.7, 4.6)
	7	3	0.0[d]	(0.0, 0.0)[d]
	All patients	49	4.2	(3.2, 4.5)
Post-treatment	1	3	6.3	(2.9, 8.6)
	2	2	N/A[c]	(1.5, 4.9)
	3	1	8.0[2]	(N/A, N/A)
	4	3	6.5	(3.9, 8.4)
	5	6	4.4	(0.2, 6.4)
	6	31	3.7	(2.2, 4.7)
	7	3	0.0[d]	(0.0, 0.0)[d]

[a] Dose level 1: Epirubicin 50 mg/m^2, Docetaxel 50 mg/m^2; Dose level 2: Epirubicin 60 mg/m^2, Docetaxel 60 mg/m^2; Dose level 3: Epirubicin 70 mg/m^2, Docetaxel 70 mg/m^2; Dose level 4: Epirubicin 75 mg/m^2, Docetaxel 75 mg/m^2; Dose level 5: Epirubicin 90 mg/m^2, Docetaxel 75 mg/m^2; Dose level 6: Epirubicin 105 mg/m^2, Docetaxel 75 mg/m^2; Dose level 7: Epirubicin 120 mg/m^2, Docetaxel 75 mg/m^2; All patients at all dose levels were given 6 mg pegfilgrastim support

[b] Value of RIN is provided ($N = 1$)

[c] Range of RIN is provided ($N = 2$)

[d] Truncation at zero

partial response, stable disease, or progressive disease (PR, SD, or PD, respectively). Only 7 of the 50 patients exhibited a pCR. Interestingly, a low maximum RIN mid-treatment (but not post-treatment) was associated with a pCR (*P* values of 0.01 and 0.28, respectively) (Table 4). Figure 3 is a plot of the maximum tumor RIN values for each patient pre-, mid-, and post-treatment. Patients are depicted according to drug dose level (colored lines) and whether they exhibited a pCR after chemotherapy (dashed lines). As shown in the figure, all patients who had a pCR post-chemotherapy exhibited a reduction in maximum tumor RIN mid-treatment. Moreover, both pCRs and mid-treatment reductions in tumor RINs were observed more commonly in patients exposed to high drug dose levels ($P = 0.04$). However, tumor RIN values increased or stayed the same post-treatment for some of these patients. Post-treatment, tumor cellularity had a significant association ($P = 0.01$) with pCR, as expected (Table 4). However, unlike RIN, there was no significant mid-treatment association between tumor cellularity and pCR ($P = 0.68$), nor with dose level at any time point (respectively, $P = 0.14, 0.77, 0.78$).

Relationship between tumor RNA integrity or tumor cellularity and the expression of specific tumor markers

Box-Cox transformations were indicated for ER [log (% positive + 0.5), to accommodate zero], PR (log), and HER2 (power –0.5); no transformations were required for tumor cellularity or Topo 2. As shown in Table 3, high baseline tumor Topo 2 levels were significantly associated with high tumor RIN pre-treatment ($P = 0.01$ (Pearson correlation), 0.03 (Spearman correlation)) and post-treatment ($P = 0.02$, 0.07). No significant association ($P > 0.05$) was observed between pre-, mid-, or post-treatment RIN and pre-treatment levels of HER2, ER, or PR (data not shown). Interestingly, there was a positive correlation between pre-treatment ER and PR levels and tumor cellularity mid-treatment (*P* values for Pearson/Spearman correlations of 0.04/0.01, and 0.01/0.01, respectively). Low post-treatment tumor cellularity was also associated with low pre-treatment PR ($P = 0.05$, for both Pearson and Spearman correlation coefficients).

Discussion

This study is one of the few clinical trials where potential biomarkers of chemotherapy response were assessed in tumors of breast cancer patients pre-, mid-, and post-treatment. Moreover, this is the first report of dose-dependent reductions in tumor RNA integrity in response to chemotherapy that are associated with treatment response. Reductions in tumor RNA integrity by epirubicin/docetaxel chemotherapy may stem from activation of tumor cell RNases [33, 34] or from the release of lysosomal RNases upon tumor cell death [33, 35]. Since both epirubicin and docetaxel are cytotoxic to endothelial cells of the tumor vasculature [36–38], their effects on tumor RNA quality may also be through nutrient/growth factor deprivation, and/or the induction of hypoxia upon destruction of tumor blood vessels. Consistent with the latter view, MCF-7 breast tumor cells cultured in the laboratory of AP for 72 h under hypoxic conditions with 9 nM epirubicin and 6 nM

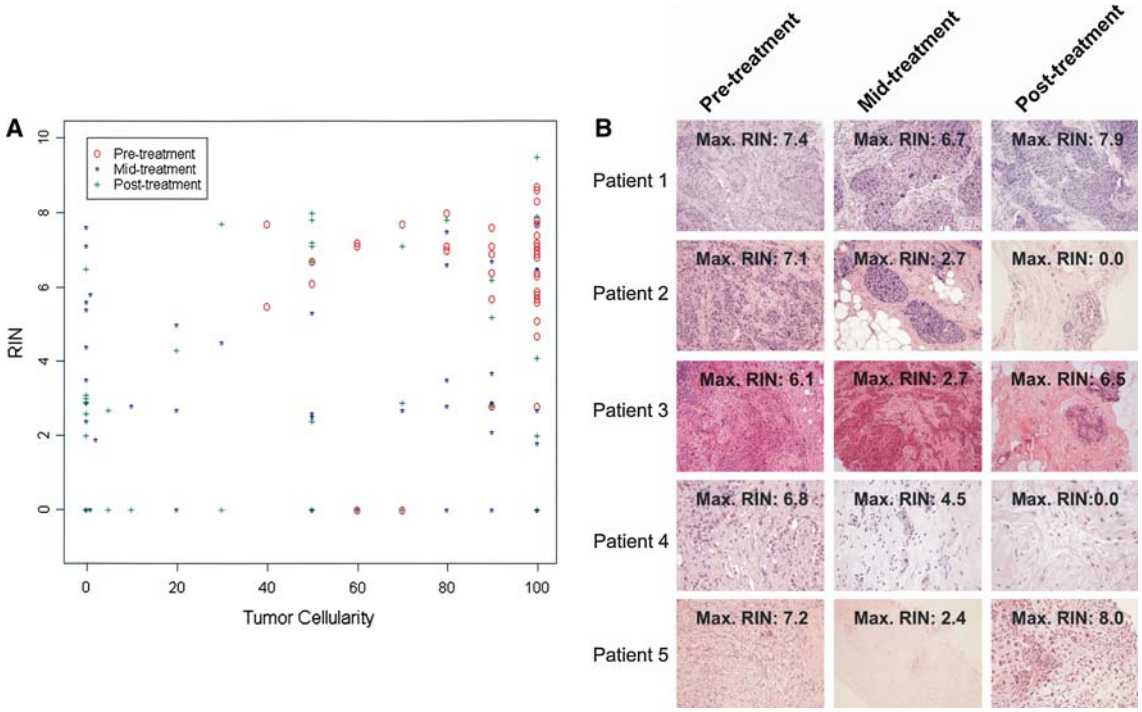

Fig. 4 Relationship between tumor RIN and tumor cellularity at various treatment times. **a** Maximum tumor RNA integrity numbers (*RINs*) are plotted against mean tumor cellularity values for image-guided core biopsies taken from MA22 patients pre-, mid-, and post-treatment with epirubicin/docetaxel chemotherapy. Photomicrographs of sections of the core biopsies from a small subset of patients used to assess tumor cellularity are depicted in *panel b* along with their corresponding RIN values

Table 3 RIN and tumor cellularity by tumor characteristics

Factor[a]	Pearson correlation	*P* value	Spearman correlation	*P* value
Tumor characteristics				
Maximum RIN and tumor cellularity[b]				
Baseline	0.15	0.29	0.09	0.55
Mid-treatment	0.06	0.69	−0.01	0.95
Post-treatment	0.52	0.0003	0.42	0.004
Maximum RIN and topo 2[c]				
Baseline	0.39	0.01	0.30	0.03
Mid-treatment	0.22	0.13	0.16	0.28
Post-treatment	0.35	0.02	0.27	0.07
Tumor cellularity and ER[c]				
Baseline	0.17	0.23	0.14	0.35
Mid-treatment	0.31	0.04	0.37	0. 01
Post-treatment	0.25	0.10	0.22	0.15
Tumor cellularity and PR[c]				
Baseline	0.20	0.17	0.12	0.42
Mid-treatment	0.39	0.01	0.36	0.01
Post-treatment	0.30	0.05	0.30	0.05

[a] Examinations between RIN and ER, PR, HER2, Topo 2 not shown: $P > 0.15$, in all instances; similarly, examinations between Tumor Cellularity and HER2, Topo 2 not shown: $P \geq 0.15$, in all instances

[b] RIN and tumor cellularity values available at all three time points: baseline, mid-treatment, post-treatment

[c] Topo 2, ER, PR values are baseline

docetaxel exhibited dramatic reductions in RNA integrity, which were not observed when cells were cultured solely under hypoxic conditions or solely in the presence of the chemotherapy agents. In contrast, the number of tumor cells was dramatically lower in the presence of epirubicin and docetaxel, with or without hypoxia (data not shown).

We also observed that mid-treatment dose-dependent reductions in tumor RIN (but not tumor cellularity) were

Table 4 RIN and tumor cellularity by response to clinical treatment

Factors assessed	Baseline P value[a]	Mid-treatment P value[a]	Post-treatment P value[a]
Maximum RIN and dose level	0.61	0.05	0.12
Maximum RIN and best clinical response (CR;PR/SD/PD)	0.12	0.61	0.64
Maximum RIN and pathologic response (CR;PR/SD/PD)	0.96	0.01	0.28
Tumor cellularity and dose level	0.14	0.77	0.78
Tumor cellularity and best clinical response (CR;PR/SD/PD)	0.86	0.95	0.91
Tumor cellularity and pathologic response (CR;PR/SD/PD)	0.72	0.68	0.01

[a] P value from one-way analysis of variance (ANOVA)

significantly associated with pCR ($P = 0.01$). Thus, tumor RIN values do not appear to simply be a surrogate measure of tumor cell number. While each pre-treatment sample with high tumor cellularity typically had a high RIN, many patients' mid- and post-treatment had high tumor RIN, but low tumor cellularity, and vice versa (Fig. 4, panel A). Tumor RIN may be a much more effective biomarker for measurement of drug response or tumor viability than tumor cellularity, since the former changed in proportion to drug dose level and was correlated with pCR earlier in treatment. In contrast, the latter may simply be a quantitative measure of the number of tumor cells regardless of their viability. Tumor cells from breast cancer patients undergoing chemotherapy may exhibit significant RNA degradation in advance of their loss of membrane integrity and destruction. In addition, since reductions in tumor RNA integrity correlated with treatment response, the RIN may serve as a useful biomarker for differentiating between drug-responsive and drug-resistant tumors.

We observed that tumor RNA quality in some patients fell during treatment, but returned to higher levels post-treatment. Two hypotheses may account for this observation. Mid-treatment, epirubicin and docetaxel may have killed the majority of tumor cells (which are drug sensitive), leaving a very small core of surviving drug-resistant tumor cells that proliferated upon continuation and conclusion of treatment. Such lesions would be expected to have high tumor cellularity and high RNA integrity post-treatment (patient 5 in Fig. 4, panel B). However, it is also possible that upon removal of chemotherapy drugs post-treatment, the lesions of patients exhibiting a pCR would be free of tumor cells and infiltrated with normal breast tissue or other cell types that would have high RNA integrity. In MA22, patient 3 exhibited this trend, where the post-treatment lesion had high RIN, groups of normal ductal cells, extensive fibrosis, and a few blood vessels, but no tumor cells. Consequently, high RNA integrity associated with lesions post-treatment need not necessarily indicate recurrent disease.

Another hypothesis to explain apparent increases in tumor RIN (and tumor cellularity) post-treatment would be differential sampling of heterogenous tumors. For example, the cores obtained from a given patient mid-treatment may have been from areas of the lesion exhibiting low tumor cellularity (high cell death), whereas samples' post-treatment may have come from areas where tumor cellularity remained high. These "sampling differences" could then account for an apparent increase in tumor cellularity or RIN post-treatment. Since RNA integrity and tumor cellularity were assessed with different core biopsies, such sampling differences could also explain the presence of tumors with low tumor cellularity but high RIN. Obtaining multiple cores throughout the lesion would minimize such sampling differences.

Since breast tumors are typically heterogeneous, the tumor RIN is a reflection of the RNA quality of all cells comprising the tumor, including any normal breast and other tissues. Image-guided core biopsies minimize the assessment of unrelated tissues. Moreover, tumor cellularity for the vast majority of patient tumors pre-treatment was very high ($90.9 \pm 2.2\%$), suggesting that tumor RIN values prior to treatment were an accurate measure of the quality of RNA in predominantly tumor cells. Moreover, our data further suggests that the strongly reduced RIN values observed mid-treatment reflected a loss of RNA and/or RNA quality specifically in tumor cells. One would therefore expect low tumor RIN values mid-treatment to be associated with treatment response. In contrast, post-treatment "tumor" RIN values may reflect the quality of RNA from both residual tumor tissue and/or normal cells that have infiltrated the lesion. Based on these observations, we would predict that patients responding to epirubicin/docetaxel chemotherapy would be those exhibiting very low tumor RIN values either mid-treatment or post-treatment (but not necessarily at both time points).

Given the increasing use of preoperative chemotherapy for the management of breast cancer, there is considerable global interest in developing strategies that will optimize

patient management. Previously, these strategies have concentrated on assessment of response using clinical examination, ultrasound, mammography and MRI. In more recent years, there has been a rapid rise in interest of looking for biological markers of chemotherapy sensitivity and resistance. The assessment of tumor RNA integrity as a biomarker of chemotherapy response could be incorporated into clinical practice by taking mid-treatment tumor core biopsies and placing them immediately in an RNA stabilization agent for rapid assessment of RNA integrity by capillary electrophoresis in a licensed laboratory. The RIN value could then be used by the clinician (along with other data) to decide whether to continue chemotherapy or proceed to other treatment options. At this point in its development, the use of tumor RNA integrity to measure chemotherapy response needs to be validated in additional clinical studies before it can be reliably incorporated into clinical practice. It is our hope that through further validation of this approach, we are on a step nearer not only to having a better understanding of in vivo response to chemotherapy but also to being able to offer a potential new tool to aid patient management in a real-time setting.

In summary, we report that tumor RNA integrity in locally advanced or inflammatory breast cancer patients is significantly decreased in a dose-dependent manner upon treatment with epirubicin/docetaxel chemotherapy. Unlike tumor cellularity, low mid-treatment maximum tumor RIN was associated with a pCR post-treatment, suggesting that tumor RIN may be serve as an early biomarker of clinical response to chemotherapy. This approach may be superior to or complement FDG-PET in measuring response to chemotherapy in breast cancer patients. Future work will be focused on validating the utility of RNA integrity as a biomarker of chemotherapy response in additional cohorts of patients, including breast cancer patients treated with other chemotherapy regimens and patients with other solid tumors or hematological malignancies.

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Conflicts of Interest/Disclosure: A PCT application has been filed by A.M.P. with the U.S. Patent Office related to the use of tumor RNA integrity as a biomarker of response to chemotherapy in cancer patients.

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