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Circulating MicroRNAs as Biomarkers of Radiation-Induced Cardiac Toxicity in Non-Small Cell Lung Cancer

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Abstract

Purpose: Radiation-induced cardiac toxicity (RICT) is an increasingly well-appreciated source of morbidity and mortality in patients receiving thoracic radiotherapy (RT). Currently available methods to predict RICT are suboptimal. We investigated circulating microRNAs (c-miRNAs) as potential biomarkers of RICT in patients undergoing definitive RT for non-small cell lung cancer (NSCLC).

Methods: Data from 63 patients treated on institutional trials were analyzed. Prognostic models of grade 3 or greater (G3+) RICT based on pre-treatment c-miRNA levels ('c-miRNA'), mean heart dose (MHD) and pre-existing cardiac disease (PCD) ('clinical'), and a combination of these ('c-miRNA + clinical') were developed. Elastic net Cox regression and full cross-validation were

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Ethics approval: All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. This study was approved by the Institutional Review Board of the University of Michigan. This article does not contain studies with animals performed by any of the authors.

Informed consent: Informed consent Informed consent was obtained from all individual participants included in the study.

used for variable selection, model building, and model evaluation. Concordance statistic (c-index) and integrated Brier score (IBS) were used to evaluate model performance.

Results: MHD, PCD, and serum levels of 14 c-miRNA species were identified as jointly prognostic for G3+ RICT. The 'c-miRNA and 'clinical' models yielded similar cross-validated c-indices (0.70 and 0.72, respectively) and IBSs (0.26 and 0.28, respectively). However, prognostication was not improved by combining c-miRNA and clinical factors (c-index 0.70, IBS 0.28). The 'c-miRNA' and 'clinical' models were able to significantly stratify patients into high- and low-risk groups of developing G3+ RICT. Chi-square testing demonstrated a marginally significantly higher prevalence of PCD in patients with high- compared to low-risk c-miRNA profile (p=0.09), suggesting an association between some c-miRNAs and PCD.

Conclusions: We identified a pre-treatment c-miRNA signature prognostic for G3+ RICT. With further development, pre- and mid-treatment c-miRNA profiling could contribute to multiparametric, patient-specific dose-selection and treatment-adaptation.

Keywords

Non-small cell lung cancer; Radiotherapy; Cardiac toxicity; Biomarker; MicroRNA

Introduction

Definitive thoracic radiotherapy (RT) for non-small cell lung cancer (NSCLC) has been linked to an increased risk of cardiac-related morbidity and mortality (Bradley et al. 2015; Chun et al. 2017; Dess et al. 2017; Wang et al. 2017). Current methods to predict RICT rely on dosimetric parameters, such as mean heart dose (MHD), and clinical factors, such as preexisting cardiac disease (PCD) (Dess et al. 2017; Wang et al. 2017). However, prediction could potentially be improved by the identification of additional biomarkers. Improved risk-stratification could be useful for guiding personalized treatment planning and dose-selection.

MicroRNAs (miRNAs) are small, non-coding RNAs that post-transcriptionally regulate gene expression (Ambros 2004). MiRNAs have been implicated in positively and negatively regulating numerous physiologic and pathologic processes, including oncogenesis and heart disease (Esquela-Kerscher and Slack 2006; Quiat and Olson 2013). MiRNAs have been shown to modulate the effects of therapeutic radiation (Cho et al. 2014; Jiang et al. 2017; Koo et al. 2017; Lan et al. 2015; Weidhaas et al. 2007; Zhai et al. 2016; Zhen et al. 2016), and in animal myocardial tissue, certain miRNA species have been implicated in promoting or protecting against RT-induced cardiac damage (Kura et al. 2016; Viczenczova et al. 2016).

While miRNAs were initially characterized in terms of their intracellular functions, extracellular, circulating miRNAs (c-miRNAs) have recently been identified in blood and other bodily fluids (Valadi et al. 2007). Like tissue miRNAs, c-miRNAs demonstrate variable expression patterns in the presence and absence of cancer, and are remarkably stable (Mitchell et al. 2008; Yuan et al. 2016). Unlike tissue miRNAs, however, c-miRNAs are readily obtainable in blood and do not require invasive tissue sampling. Given these features, c-miRNAs represent attractive candidates for biomarker development (Kong et al. 2012).

While c-miRNAs have shown promise as biomarkers of cardiac disease (Matsumoto et al. 2013; Wang et al. 2016; Zampetaki et al. 2012), as well as response to and survival following RT for NSCLC (Chen et al. 2016; Sun et al. 2017), their utility in predicting RICT has not been explored.

Due to the clinical challenge posed by RICT and the lack of patient-specific biomarkers for risk stratification, we sought to identify a pre-treatment c-miRNA signature for prediction of RICT in patients treated with definitive RT for locally advanced and medically inoperable NSCLC.

Materials and methods

Patient cohort

We analyzed data from 63 patients treated on four prospective Institutional Review Board-approved lung-cancer studies: (1) a phase 1–2 study of radiation dose-escalation with concurrent chemotherapy (NCT not available), (2–3) two consecutive studies using functional imaging and biomarkers to assess patient outcome (NCT not available and NCT00603057), and (4) a study using mid-treatment positron emission tomography (PET) to guide individualized dose-escalation (NCT01190527). NCT numbers are not available for the earliest two trials as they were not registered with clinicaltrials.gov. Primary trial outcomes are not reported in this manuscript, but data from patients treated on these protocols were included in this analysis. Patients with stage II-III NSCLC and a Karnofsky performance status (KPS) of at least 60 were included in this study, whereas patients with a component of small-cell lung cancer and those treated with stereotactic body RT (SBRT) were excluded.

Treatment regimen

All patients were treated with definitive RT, with or without concurrent chemotherapy. After completion of RT, some patients were also treated with consolidative chemotherapy, depending on their respective protocol and physician preference. Target radiation doses ranged from 66 to 86 Gy in daily fractions, as directed by the respective protocols. Radiation was delivered using three-dimensional conformal RT (3DCRT) as previously described (Kong et al. 2005). Gross tumor volume included the primary tumor and any involved regional lymph nodes, as determined by tissue diagnosis and/or positron emission tomography (PET). Uninvolved lymph node regions were not included in the clinical target volume. Tissue inhomogeneity corrections were applied for all plans. Heart constraints employed in these trials included volume receiving 40 Gy < 100% (V40Gy < 100%) and V65Gy < 33%.

As dose and fractionation varied among patients, we standardized values to equivalent doses in 2-Gy fractions (EQD2) (Fowler 1989). EQD2 values were calculated using the linear-quadratic formula with an alpha-beta ratio of 10 Gy for tumor and 2.5 Gy for heart (Hall and Giaccia 2006).

Sample collection, RNA isolation, and miRNA profiling

Blood samples were collected using red-top tubes with no use of anticoagulant within one week prior to initiation of thoracic radiation, following which they were processed as previously described (Sun et al. 2017). Total RNA was isolated from serum samples using the miRNeasy Mini Kit (Qiagen, Hilden Germany) following the manufacturer's protocol with slight modifications, as previously described (Sun et al. 2017). Post-isolation, RNA concentration and quality were determined using a Nanodrop 2000 instrument (Thermo Scientific, Waltham, Massachusetts).

Serum RNA was reverse transcribed to complementary DNA (cDNA) using the miScript II Reverse Transcription Kit (Qiagen, Hilden Germany). Reverse-transcription products were analyzed for the presence and differential expression of a panel of 62 miRNAs detectable in serum, plasma, and other bodily fluids using Human Serum & Plasma miRNA PCR Arrays (Cat. No MIHS-106Z, Qiagen, Hilden Germany) as previously described (Philippidou et al. 2010; Ryu et al. 2011). A list of all miRNA species assayed is found in Supplemental Table 1. In order to eliminate potential variation introduced during the isolation and RNA quantification processes, the raw Ct value for each miRNA was normalized to the raw Ct value for spike-in cel-miR-39 obtained from each individual sample using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). C-miRNA levels are described as elevated or depressed relative to the mean value of each cmiRNA calculated from the entire patient population.

Outcome definitions

The primary endpoint was grade 3 or greater (G3+) RICT, modeled as a survival-type endpoint to account for differential follow-up. Patients alive at their last follow-up or who died of causes unrelated to RICT were censored at those dates. Radiation-induced cardiac toxicity was initially graded per CTCAE v.3.0 and then for this analysis reviewed, confirmed, and updated to CTCAE v4.03. In addition, cardiac events not previously attributed to radiation were documented, graded, and included in the analysis. All events were confirmed by two independent physicians without knowledge of the treatment plan or cardiac radiation dose.

Statistical methods

We investigated mean heart dose (MHD), pre-existing cardiac disease (PCD), and pre-treatment serum c-miRNA levels as potential prognostic biomarkers of G3+ RICT. Mean heart dose and PCD were selected as they had been previously identified by our group as significant contributors to the development of RICT (Dess et al. 2017). Pre-existing cardiac disease was defined as the presence of either ischemic heart disease or congestive heart failure (CHF). Ischemic heart disease was defined as acute myocardial infarction (MI); coronary artery bypass grafting procedure, angioplasty or stent placement; or a diagnosis of coronary artery disease (CAD).

Cox elastic net regression was used to model the risk of G3+ RICT as a function of covariates. Elastic net is a hybrid of LASSO and RIDGE regression techniques and retains the good prediction performance of RIDGE while also enforcing sparsity (Zou and Hastie

2005). All variables were modeled as main effects. Three separate models were fit: one consisting of cmiRNA signature ('c-miRNA'), one consisting of MHD and PCD ('clinical'), and one consisting of a combination of these ('c-miRNA + clinical'). Factors were combined into these models using Cox elastic net multivariable regression with the relative weight of each factor being related to their respective coefficients derived from model fitting. The c-miRNA signature combined the effects of each selected c-miRNA into an aggregate estimate of c-miRNA-attributable risk. Model performance was assessed by concordance statistic (c-index) (Heagerty and Zheng 2005) and integrated Brier score (IBS) (Graf et al. 1999). The c-index describes a model's discriminatory ability in a manner similar to area under the receiver operating characteristic curve (AUC), but differs from AUC in that it considers the time to an event instead of cumulative incidence at a specified time point. A higher c-index indicates superior discriminatory ability. The Brier score measures the squared difference between predicted and observed survival with censorship. The integrated Brier Score (IBS) is a summary of the cumulative prediction error integrated over a time period. A lower IBS denotes superior model performance, with an IBS of 0 indicating perfect model predictions over the period of interest. IBS is commonly used as a performance score to evaluate the predictive performance of survival models (Rufibach 2010; Schumacher et al. 2003).

Model assessment and tuning parameter selection were performed using 5-fold cross validation repeated 20 times (to minimize variability associated with fold choice). Each time, the four training-fold dataset was used to build the prognostic model (including data normalization, fitting Cox elastic net), following which the corresponding test-fold dataset was used for cross-validated c-index and IBS estimation.

In order to investigate the ability of these models to risk-stratify in terms of RICT, we calculated Kaplan-Meier estimates of the proportion of patients experiencing G3+ RICT over time in low- versus high-risk patient groups. Risk grouping was determined from repeated stratified 5-fold cross validation with the resultant median being used as dichotomization point. To assess the relationship between c-miRNA group and PCD, we performed two way table chi-square testing, using Monte Carlo simulation to calculate the corresponding p-value. All statistical analyses were performed with R version 3.2.1.

Results

Characteristics of the 63 patients analyzed in this study are found in Table 1. Of all patients, 11 developed G3+ RICT, corresponding to a cumulative rate of 16.9%. Six of these events were acute coronary syndrome (ACS), two were CHF, two were cardiac arrest, and one was arrhythmia.

In addition to MHD and PCD, multivariable elastic net analysis identified 14 c-miRNA species for which variations in serum concentrations correlated with subsequent development of G3+ RICT (Table 2). Of these, higher levels of five were associated with increased risk, while higher levels of 9 were associated with decreased risk. The c-miRNA associated with the greatest hazard ratio (HR) for G3+ RICT was miR-574–3p, for which an elevation in serum levels one standard deviation above the mean was associated with a HR of 1.85. MiR-15b and miR-21, two miRNA species previously implicated in regulating

cardiac response to RT in animal studies (Kura et al. 2016; Viczenczova et al. 2016), were not significantly correlated with G3+ RICT in this analysis and were therefore not included in the subsequent modeling (Supplemental Table 2).

We next generated three models for prognostication of G3+ RICT based on c-miRNA signature ('c-miRNA'), MHD and PCD ('clinical'), and a combination of these ('c-miRNA + clinical'). Table 3 lists the cross-validated c-index and IBS associated with each model. The highest c-index, corresponding to superior discriminatory ability, was obtained using the 'clinical' model, and the lowest IBS, corresponding to superior overall performance, was obtained using the 'c-miRNA' model, although differences in these values among models were modest. In spite of the fact that multivariable elastic net analysis identified all of these factors as adding value to G3+ RICT prediction, combining c-miRNA and clinical factors did not improve model performance.

As no p-values are available from penalized regression techniques such as elastic net, we next sought to empirically test whether the models could distinguish between patients who are at lower or higher risk of G3+ RICT. To do this, we used each model to divide patients into low- and high-risk groups and analyzed Kaplan-Meier estimates of G3+ RICT in each group. Hazard ratios of G3+ RICT in low- versus high-risk patients were 0.25 (p=0.04, 95%CI 0.06–0.94), 0.17 (p=0.02, 95%CI 0.04–0.79), and 0.29 (p=0.07, 95%CI 0.08–1.12) for the 'c-miRNA,' 'clinical,' and 'c-miRNA + clinical' models, respectively, indicating significant risk-stratification by the 'c-miRNA' and 'clinical' models. The corresponding Kaplan Meier curves are shown in Figure 1.

Because adding c-miRNA data to clinical factors did not result in an improved c-index or IBS, we hypothesized that the c-miRNA signature may to some degree be correlated with PCD. To investigate this, we performed chi-square comparison of the prevalence of PCD in patients designated high- vs. low risk by the 'c-miRNA' model (Table 4). This resulted in a p-value of 0.09, indicating a marginally significant association between pre-treatment c-miRNA signature and PCD, and suggesting that this association may partially account for the prognostic ability of the c-miRNA signature.

Discussion

In this work, we identified a pre-treatment c-miRNA signature prognostic for G3+ RICT in patients treated with definitive RT for stages II-III NSCLC. This signature was comprised of 14 cmiRNA species for which variations in serum concentrations correlated with increased or decreased risk of G3+ RICT. A model based on this c-miRNA signature performed as well as a model based on MHD and PCD. Although multivariable analysis indicated that this c-miRNA signature added prognostic value to MHD and PCD, combining these factors into a 'c-miRNA + clinical' model did not improve accuracy. Both the 'c-miRNA' and 'clinical' models were able to significantly stratify patients as low- or high-risk for development of G3+ RICT.

The incidence of G3+ RICT in this study was 16.9%, which is higher than reported in many previous trials. For example, the rates of acute and late G3+ cardiac events among all

patients in RTOG 9410 were approximately 2% and 2.5%, respectively (Curran et al. 2011). The relatively high incidence in the present study is likely due to multiple factors. One important consideration is that many of these patients were treated with dose-escalated RT. In other recent analyses of dose-escalated RT for NSCLC, rates of cardiac toxicity have been comparable. For example, in RTOG 0617, the rate of G3+ cardiac toxicity was 24% (Speirs et al. 2017). In a retrospective analysis of dose-escalated RT from the University of North Carolina, the rate of symptomatic cardiac events was 23% (Wang et al. 2017). Multiple studies have shown a correlation between cardiac dose and toxicity (Dess et al. 2017; Wang et al. 2017). Another contributing factor may have been accuracy of toxicity reporting. Some have suggested that cardiac toxicity is commonly under-reported in clinical trials employing thoracic RT (Faivre-Finn 2015; Gaya and Ashford 2005). In this study, thorough review medical records disclosed additional cardiac events beyond those captured during protocol-driven data collection.

The two factors incorporated in our clinical model, MHD and PCD, were selected because they have been previously shown by our group to be highly prognostic for cardiac-related morbidity and mortality following RT for NSCLC (Dess et al. 2017). In this analysis, these factors were again prognostic of G3+ RICT. The observation that combining c-miRNA data with these variables did not improve prognostication suggests that certain of these factors may be correlated with each other. As a correlation between MHD and pre-treatment c-miRNA profile seemed unlikely, we hypothesized that at least some of the c-miRNAs identified may be associated with PCD. Chi-square testing of observed versus expected prevalence of PCD in patients defined as high-risk by c-miRNA profile yielded a marginally significant p-value of 0.09. This result suggests that the mechanism by which some, but not all, of the c-miRNA species prognosticate for G3+ RICT is through their association with PCD.

The hypothesis that a c-miRNA signature could be associated with PCD is supported by previously published studies. There are numerous reports of various miRNA species functioning as biomarkers of cardiac disease, including some investigating the same miRNAs identified in this work. For example, circulating and/or tissue levels of miR-100, −106b, −145, −146, and −223, have been observed to inversely correlate with MI, CAD, CHF, atherosclerosis, type 2 diabetes, obesity, and atrial fibrillation (Chiang et al. 2014; Faccini et al. 2017; Liu et al. 2012; Pek et al. 2016; Ramkaran et al. 2014; Weber et al. 2011; Yang et al. 2011; Zampetaki et al. 2012). In the current study, higher serum levels of these c-miRNAs were associated with lower risk of G3+ RICT, which is consistent with the referenced works. In addition, miR-134, −200b, −574, −858, and let-7c levels have been correlated with ACS, MI, CAD, atherosclerosis, CHF, and cardiomyopathy (Ghosh et al. 2012; He et al. 2014; Ikeda et al. 2007; Lan et al. 2016; Vogel et al. 2013; Wang et al. 2016; Zhou et al. 2015). In this study, higher serum levels of these miRNAs were associated with higher risk of G3+ RICT, which is also consistent with the cited works. However, the relationships of some of the c-miRNAs identified do not coincide with published studies. These include miR-192 and −195, higher levels of which have been correlated with heart failure and MI (Long et al. 2012; Matsumoto et al. 2013; van Rooij et al. 2006). In our study, however, higher levels of these c-miRNAs were associated with lower risk of G3+ RICT. Taken together, these comparisons suggest that the prognostic utility of some of the

identified c-miRNA species may be related to their correlation with PCD, but that others may be associated with RICT through different mechanisms. Further study of these miRNA species is needed in order to more fully understand how they may function as biomarkers of RICT or cardiac disease of other etiologies.

Although the c-miRNA model was associated with the lowest cross-validated IBS, it only slightly outperformed the clinical model in this regarded and was associated with a slightly inferior cross-validated c-index. As such, the clinical utility of this c-miRNA signature in its current form is likely limited. However, there are several important implications of this work. For example, many of the miRNA species identified in this study appear to be involved in promoting or protecting against cardiac disease. It should be noted, however, that it is difficult to draw conclusions regarding the biologic function of miRNAs from biomarker studies. For example, in a hypothetical patient with cardiac disease, serum levels of a given miRNA could be elevated either because it is directly contributing to cardiac damage or because its expression has been induced as a compensatory mechanism of attempted repair. Further investigation of these miRNAs may expand our understanding of the roles miRNAs play in cardiac disease of multiple etiologies and uncover targets for therapeutic intervention. In addition, while in this study we investigated pre-treatment c-miRNA data, it is possible that mid-treatment changes in c-miRNA levels may provide additional prognostic information. Cardiac irradiation may elicit miRNA-mediated responses that vary among patients, with up- or down-regulation of various species being associated with lower or higher risk of RICT. These changes may be detectable prior to the corresponding clinical manifestations of RICT. The c-miRNAs identified in this study represent attractive candidates for study as mid-treatment biomarkers of cardiac damage. With further study, a mid-treatment c-miRNA profile could be identified that could guide adaptation of RT and/or inform post-RT optimization of cardiac health.

There are limitations to this study that should be considered. While we performed rigorous cross-validation of parameter selection and model performance, we have not externally validated our findings. The study was also constrained by the limited number of c-miRNA species measured. Samples were analyzed at a time when miRNA profiling technologies were less comprehensive than those currently available. In addition, the miRNAs measured were not selected based on possible roles in cardiac function. As a result, certain miRNAs implicated by others in cardiac disease were not evaluated, such as miRNA-1 (Kura et al. 2016; Viczenczova et al. 2016). Future studies should evaluate broader panels of miRNAs in order to increase chances of identifying useful prognostic factors.

Radiotherapy for locally-advanced NSCLC is complicated by competing goals to maximize disease control while minimizing toxicity. This challenge is particularly evident when selecting target dose and normal-tissue constraints during RT planning. An ideal method to guide this process would employ multiple patient-specific factors, as opposed to parameters derived from population-based studies. To develop an individualized, multiparametric approach, clinical, biological, imaging, and other biomarkers of response and toxicity must be characterized. This work represents significant progress toward this aim. With further improvement, the c-miRNA signature described here could contribute to a powerful model for prediction of RICT. Such a model could, in turn, be utilized in conjunction with

additional factors predictive of other outcomes to comprehensively direct personalized treatment planning. In addition, study of mid-treatment changes in these and other biomarkers could allow for treatment adaptation to further individualize RT.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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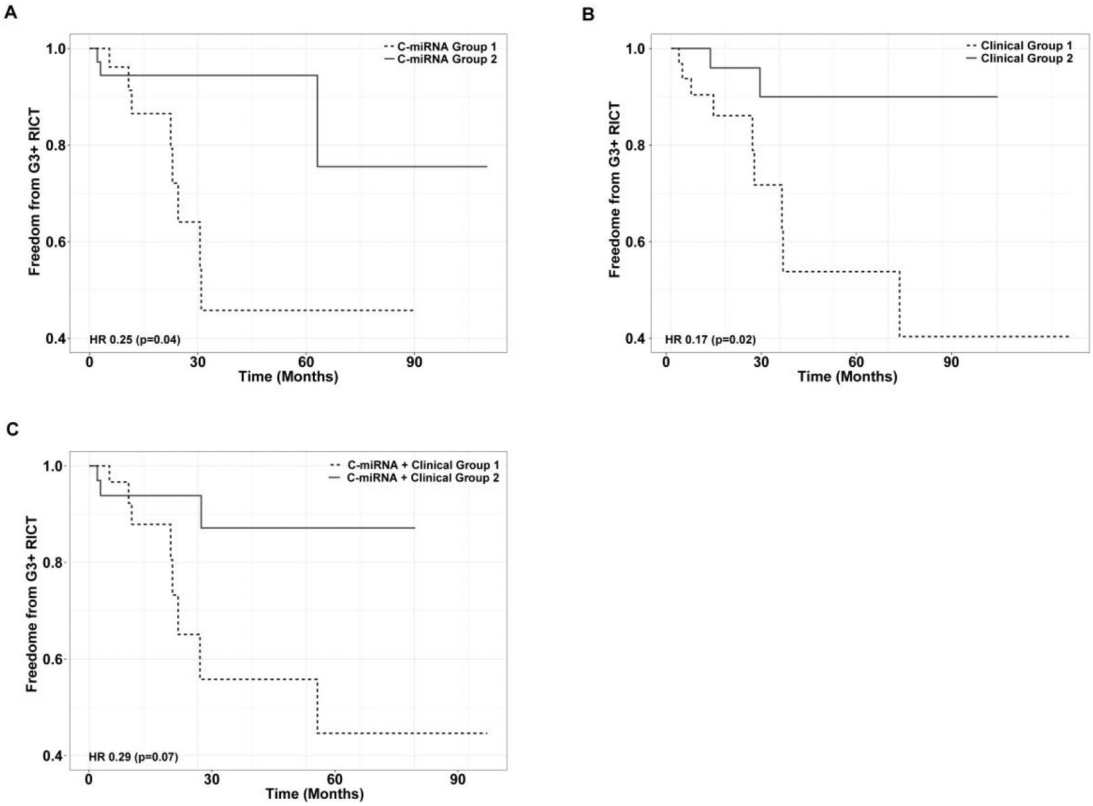

Figure 1. Kaplan-Meier estimates of G3+ RICT in low- and high-risk patients as determined by c-miRNA (A), clinical (B), and c-miRNA + clinical (C) models.

G3+ = grade 3 or greater. RICT = radiation-induced cardiac toxicity. HR = hazard ratio.

Table 1.

Demographic, disease, and treatment characteristics of study participants.

Characteristic	Value
Median age, years (range)	65.7 (45.3 – 84.6)
Sex, n (%)	
Female	15 (23.8%)
Male	48 (76.2%)
KPS, n (%)	
81 – 100	32 (50.8%)
71 −80	21 (33.3%)
60–70	10 (15.9%)
Smoking status, n (%)	
Current	32 (50.8%)
Former	29 (46.0%)
Never	2 (3.2%)
Unknown	0
PCD, n (%)	
Yes	19 (30.2%)
No	44 (69.8%)
Tumor histology, n (%)	
Adenocarcinoma	20 (31.7%)
Squamous cell carcinoma	21 (33.3%)
NSCLC NOS	22 (34.9%)
Group stage, n (%)	
11A	2 (3.2%)
MB	5 (7.9%)
IIIA	24 (38.1%)
IIIB	32 (50.8%)
Concurrent chemotherapy, n (%)	
Yes	53 (84.1%)
No	10 (15.9%)
Mean radiation dose, Gy (EQD2)	
Mean target dose (Std Dev)	74.4 (8.7)
Mean MHD (Std Dev)	13.7 (8.8)

KPS = Karnofsky performance status. PCD = pre-existing cardiac disease. NSCLC = non-small cell lung cancer. NOS = not otherwise specified. Gy = Gray. EQD2 = equivalent dose in 2-Gy fractions. Std Dev = standard deviation. MHD = mean heart dose.

Table 2.

Coefficients and hazard ratios (HRs) associated with the 16 variables selected by elastic net as prognostic for G3+ RICT.

Variable	Coefficient (log(HR))	HR
PCD	1.113	3.043
MHD (per 10 Gy)	0.180	1.197
miR-100–5p	−0.006	0.994
miR-106b-5p	−0.080	0.924
miR-145–5p	−0.176	0.839
miR-146a-5p	−0.598	0.550
miR-192–5p	−0.282	0.754
miR-195–5p	−0.150	0.861
miR-223–3p	−0.033	0.968
miR-25–3p	−0.191	0.826
miR-34a-5p	−0.325	0.722
miR-574–3p	0.616	1.851
miR-885–5p	0.499	1.647
Iet-7c	0.284	1.328
miR-200b-3p	0.501	1.651
miR-134	0.086	1.089

HR for miRNAs represents change in risk associated with in 1 crease in serum concentration 1 standard deviation above the mean value for that miRNA among all patients.

HR = hazard ratio. PCD = pre-existing cardiac disease. MHD = mean heart dose.

Table 3.

C-indices for prognostication of G3+ RICT using indicated models.

	c-index	IBS
C-miRNA	0.70	0.26
Clinical Factors	0.72	0.28
C-miRNA + Clinical Factors	0.70	0.28

C-index = concordance statistic. IBS = integrated Briar score.

Table 4.

Chi-square table of PCD in patients with high- vs. low-risk c-miRNA profile.

	PCD		p value
	Yes	No	
c-miRNA Group 1	13	19	0.09
c-miRNA Group 2	6	25	

PCD = pre-existing cardiac disease.