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A Gene Expression Model of Intrinsic Tumor Radiosensitivity: Prediction of Response and Prognosis after Chemoradiation

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Conflict of Interest: SE and JTR are named as inventors in a patent application of the technology described.

Abstract

Purpose: Development of a radiosensitivity predictive assay is a central goal of radiation oncology. We reasoned a gene expression model could be developed to predict intrinsic radiosensitivity and treatment response in patients.

Methods and Materials: Radiosensitivity (determined by survival fraction at 2 Gy) was modeled as a function of gene expression, tissue of origin, ras status (mut/wt) and p53 status (mut/wt) in 48 human cancer cell lines. 10 genes were identified and used to build a rank-based linear regression algorithm to predict an intrinsic radiosensitivity index (RSI, high index=radioresistance). This model was applied to three independent cohorts treated with concurrent chemoradiation: 1. Head and neck cancer (HNC, n=92) 2. Rectal cancer (n=14) 3. Esophageal cancer (n=12).

Results: Predicted RSI was significantly different in responders (R) vs. non-responders (NR) in the rectal (RSI R vs. NR 0.32 vs. 0.46, p=0.03), esophageal (RSI R vs. NR 0.37 vs. 0.50, p=0.05) and combined rectal/esophageal (RSI R vs. NR 0.34 vs. 0.48, p=0.001511) cohorts. Using a threshold RSI of 0.46, the model has a sensitivity of 80%, specificity of 82% and positive predictive value of 86%. Finally, we evaluated the model, as a prognostic marker in HNC. There was an improved 2yr locoregional control (LRC) in the predicted radiosensitive group (2yr LRC 86% vs. 61%, p=0.05).

Conclusions: We validate a robust multi-gene expression model of intrinsic tumor radiosensitivity in three independent cohorts totaling 118 patients. To our knowledge, this is the first time that a systems-biology based radiosensitivity model is validated in multiple independent clinical datasets.

Keywords: Intrinsic radiosensitivity; gene expression; predictive assay; chemoradiation; systems biology

Introduction

Personalized medicine holds the promise that the diagnosis, prevention and treatment of cancer will be based on individual assessment of risk [1]. Significant advances towards personalized radiation therapy (RT) have been largely achieved by physical advances in radiotherapy treatment planning and delivery [2]. In contrast, the efforts in understanding the biological parameters that define intrinsic radiosensitivity have not met the same success. Thus, RT is prescribed without considering the potential individual differences in tumor and patient radiosensitivity. However there is evidence to suggest that differences in intrinsic radiosensitivity exist [3] and understanding their biological basis could significantly impact clinical practice. Thus, a successful radiosensitivity predictive assay would be central to the development of biologically-guided personalized treatment strategies in radiation oncology. However, although a number of promising approaches have been developed in the past (1. determination of ex-vivo tumor SF2[4-6] 2. electrodes to measure tumor hypoxia, [7] 3. determination of tumor proliferative potential (Tpot) [8, 9]), none has become routine in the clinic.

The advent of high dimensional and high-throughput technologies have provided an opportunity to address the development of biomarkers from a different perspective. For example, gene expression signatures have been shown to be prognostic in breast, lung, and HNC cancer [10-12]. Further, recent studies have identified biomarkers predictive of patient response to drug treatment [13]. Moreover, RT may represent a common denominator in cancer therapeutics,

since approximately 60% of cancer patients are treated with RT [14]. We have previously shown that gene expression can predict cellular intrinsic radiosensitivity[15]. In addition, we developed a systems biology model of radiation sensitivity that identified ten hub genes (see companion paper). We reasoned a gene expression model could be developed to predict radiosensitivity in patients from these hub genes.

In this paper we apply a novel multi-gene expression model of intrinsic tumor radiosensitivity. The model is based on the expression of ten hub genes identified by the systems biology model of radiosensitivity. This model predicts a radiosensitivity index (RSI) that is directly proportional to tumor radioresistance. We clinically validate the model as a predictive factor of pathological response in two independent cohorts of esophageal (n=12) and rectal (n=14) cancer patients treated with preoperative concurrent chemoradiation in prospective clinical trials at Moffitt Cancer Center. In addition, we find RSI is of prognostic value in a third external dataset of HNC cancer patients (HNC, n=92) treated with definitive concurrent chemoradiation within Phase 2 and 3 clinical trials at the Netherlands Cancer Institute. In conclusion, we think this model may play a central role in individualizing therapy in radiation oncology.

Methods and Materials

Rectal Cancer Cohort –14 patients were enrolled in an IRB-approved prospective Phase 1 trial evaluating escalating doses of oral topotecan as a radiosensitizing agent. Informed consent was obtained prior to enrollment. Eligibility criteria included patients with histologically-confirmed rectal cancer, a primary tumor ≥ 3 cm, clinical stage ≥ T_2 and ECOG performance status < 2. All subjects were treated at Moffitt Cancer Center and were clinically-staged by endoscopic ultrasound (EUS). Tumor biopsies (5 core biopsies) were obtained before initiation of therapy and snap frozen in liquid nitrogen. No tumor macro or microdissection was performed.

All subjects were treated with preoperative concurrent radiochemotherapy and underwent surgical resection (APR or LAR in 13/14) within 8 weeks of completion of preoperative treatment. The starting dose of oral Topotecan was 0.25 mg/m2 and it was administered at least 3 hours before RT on a daily basis. Patients were treated to 45 Gy to a standard pelvic field (three or four field 3-D conformal technique). Table 1 shows the clinical characteristics of this cohort.

Response Definition (Rectal Cancer) – Pathological Response was defined by at least a decrease of one T stage in the primary tumor between the pretreatment EUS and the pathological evaluation of the specimen [16, 17]. Pathological complete response was defined as no evidence of tumor in the surgical specimen (primary

and nodes). Based on this definition, 57% (8/14) of the patients were considered responders.

Esophageal Cancer Cohort –12 patients were enrolled in an IRB-approved prospective tissue collection trial, aimed at defining prognostic molecular signatures in esophageal cancer. Clinical management was not dictated by the protocol and left to the clinical judgment of the treating physicians. Treatment details are presented in table 2. Eligibility criteria included a histological diagnosis of esophageal cancer, deemed a reasonable candidate for preoperative radiochemotherapy and/or esophagectomy, an ECOG performance < 2 and chemotherapy-naïve. Subjects were clinically staged by EUS. All tumor biopsies were obtained before treatment and snap frozen in liquid nitrogen. No macro or microdissection was performed.

Subjects were treated with concurrent radiochemotherapy to be followed by planned esophagectomy. 9/12 underwent planned esophagectomy. Three patients completed concurrent radiochemotherapy but were not operated because of patient or physician preference (2 patients) or progressive disease (one patient). The clinical characteristics of this cohort is summarized in Table 2

Response Definition (Esophageal Cancer) – This was defined as a decrease of at least two T stages between the pretreatment EUS evaluation and the pathological evaluation of the specimen [18]. Three patients did not undergo

esophagectomy. One had progressive disease during preoperative therapy; and two experienced clinical complete responses (documented by PET and/or EUS and biopsy) and had no evidence of disease at least one year after completion of treatment. Because pathologic information was not available, response for these three patients was defined on clinical criteria. The patient with progressive disease was deemed a non-responder, while the two patients with documented clinical complete responses and at least one year of follow up were deemed responders. Based on this definition 50% (6/12) of the patients were considered responders.

HNC Cohort –92 patients were treated within prospective randomized Phase II - III trials at The Netherlands Cancer Institute [19]. Tumors were mostly locally-advanced (94% T3 and above, 74% N1 and above). The full clinical details of this cohort were previously published[19]. All patients were treated with concurrent radiochemotherapy with cisplatin-based chemotherapy. Radiation dose was 70Gy in 2Gy fractions. Three schedules of cisplatin were given: 1. 100mg/m^2 IV three times during radiotherapy 2. 150 mg/m^2 given intra-arterially four times during radiotherapy 3. 20 x 6mg/m^2 daily. No disease outcome differences were found between chemotherapy schedules.

Microarrays (rectal and esophageal cohorts) – Total RNA was isolated using the TRIzol Reagent (Invitrogen, Carlsbad, CA) and the manufacturer's protocol

and purified using the RNeasy cleanup procedure (Qiagen Inc.,Valencia, CA). RNA quality was assessed by gel electrophoresis , $A_{260}/_{280}$ ratio or by the Agilent 2100 Bioanalyzer. Five micrograms of total RNA was processed. The poly(A) RNA was converted to cDNA, amplified and labeled with biotin[20]. Hybridization with the biotin labeled RNA, staining, and scanning was performed as described[21]. Affymetrix U133Plus2.0 chips were normalized using the robust multi-array analysis method [22]

Microarrays (HNC cohort) – These methods were previously published [19]. Briefly, pretreatment biopsies were taken during examination under general anesthesia and snap frozen in liquid nitrogen. Biopsies containing >50% tumor cells were used. Extracted RNA was quality controlled by Bioanalyzer, amplified, labeled and hybridized to 70-mer oligo micorarrays containing 34,580 probes representing 24,650 genes (Operon v.3.0)

10 Gene Systems Model: The model was developed in 48 cancer cell lines from the NCI panel of 60. Radiosensitivity measurements (SF2) were either determined in our lab (25 cell lines) or obtained from the literature (23 cell lines). Gene expression was from Affymetrix HU6800 Genechips from a previous study [23] and were preprocessed using MAS 5.0.

We developed a systems model of radiosensitivity by expanding a previously-validated model [15] to include biological variables known to influence radiophenotype: tissue of origin (TO), ras status (mut vs. wild-type) and p53 status (mut vs. wild-type). This analysis is given by the following equation (Supplemental Tables 1-4):

$$SF2_x = k_0 + k_1(y_x) + k_2(TO) + k_3(\text{ras status}) + k_4(\text{p53 status}) + k_5(y_x)(TO) + k_6(y_x)(\text{ras status}) + k_7(TO)(\text{ras status}) + k_8(y_x)(\text{p53 status}) + k_9(TO)(\text{p53}) + k_{10}(\text{ras status})(\text{p53 status}) + k_{11}(y_x)(TO)(\text{ras status}) + k_{12}(y_x)(\text{ras status})(\text{p53 status}) + k_{13}(TO)(\text{ras status})(\text{p53status}) + k_{14}(y_x)(TO)(\text{ras status})(\text{p53 status}) \ldots$$

The model consisted of all non-singular terms (28 terms) including gene expression (y_x), p53 mutation status, ras mutation status, TO and all possible interactions among terms (Supplemental Table 5). TO, p53 mutation and ras mutation status are categorical variables and were coded as dummy variables. This analysis is performed on a gene by gene basis, totaling 7,168 probesets. The 500 gene-based models with the smallest sum of squared residuals (best linear fit) were selected for further analysis (Supplemental Table 6).

The 500 selected genes were uploaded into the GeneGO™ MetaCore™ software (GeneGO, Encinitas, CA) and the primary edges (interconnections) were plotted using literature-based annotations and the model was reduced by

identifying all genes (network hubs) with more than 5 edges and less than 50% of edges hidden within the network (Supplemental Table 7). This resulted in the 10 genes described in this paper. The Gather program was used to identify significant relationships of terms from the 10 genes (threshold p<0.005).

Predictive Model Development –Gene expression for the 10 genes were rank-ordered, with lowest expression ranked 1. Radiosensitivity was modeled using a linear regression model of the ten genes in the 48 cell lines using the R software (see results for equation). This model was applied to similarly rank-ordered patient data to generate the RSI.

Statistical Analyses – A one-sided Mann-Whitney test was used to determine if the predicted RSI was significantly higher for non-responders. Bar-charts of patient response were graphed using mean and standard error values for each response group. Locoregional recurrence was defined previously [19]. Locoregional control differences between low and high RSI values were calculated using the log-rank test. Since the model does not account for the radiosensitizing effect of chemotherapy, we expected the model would be most accurate in the most radiosensitive quartile. Thus, the RSI cut-point for HNC patients was pre-defined at the 25th percentile.

Microarray Platform Translation – Gene Mapping - Probes were mapped from the HU6800 platform to the HG-U133 Plus 2.0 platform and NKI array

format by mapping the probe sequences onto a corresponding NCBI refseq ID or genomic region, then identifying the closest probe match on the new microarray platform (Table 3).

Results

A radiosensitivity systems model captures central regulatory pathways in radiation response

Table 3 shows the ten "hub" genes on which expression the radiosensitivity model is built. The selected genes are biologically-important and are involved in regulating radiation signaling [24-32]. In addition, 7/10 (*HDAC1*, *PKC-beta*, *RelA*, *c-Abl*, *STAT1*, *AR*, *CDK1*) have been studied as targets for radiosensitizer development [32-37]. Furthermore, the Gene Ontology (GO) terms captured by the 10 gene systems model, include DNA damage response, histone deacetylation, cell cycle regulation, apoptosis and proliferation, all of which play an important role in radiation response [34, 38, 39]. In summary, the systems model captures central pathways and genes involved in regulating radiosensitivity.

Development of a radiosensitivity predictive model based on the systems model

We developed and optimized a linear regression algorithm to predict radiosensitivity, using gene expression of the 10 hub genes in the systems model. Translation of the model to other datasets was an important requirement, therefore the hubs were assigned ranks by expression and the linear regression model was built from ranks (instead of absolute expression) [40]. The model predicts a continuous RSI that is based on the survival fraction at 2Gy (SF2), measured for the cell lines in the database. Thus, RSI is directly proportional to radioresistance (high index=radioresistance). Since the 10 hubs were selected from the cell line data, an estimate of accuracy generated by cross-validation of the model in the same cell lines would yield optimistically-biased estimates of accuracy. Therefore, we used additional datasets for validation (clinical datasets below). The rank-based linear regression equation is the following: RSI=-0.0098009*AR + 0.0128283*cJun + 0.0254552*STAT1 - 0.0017589*PKC - 0.0038171*RelA + 0.1070213*cABL – 0.0002509*SUMO1 – 0.0092431*CDK1 - 0.0204469*HDAC – 0.0441683*IRF1

The radiosensitivity model predicts pathological response to chemoradiation in rectal and esophageal cancer

We applied the pre-defined model to the prediction of clinical response to concurrent radiochemotherapy in two independent prospectively-collected pilot cohorts of patients with rectal (n=14) and esophageal cancer (n=12). Pathological response was defined by T stage criteria (see methods). It should be emphasized that all features in the model were pre-defined, including the ten genes, the rank-based linear regression approach and the coefficients. The model significantly separated responders (R) from non-responders (NR) in the pilot clinical cohort (Figure 1) (all patients, mean predicted RSI, R vs. NR 0.34 vs. 0.48, p=0.002). Importantly, the model was accurate in both disease cohorts despite the small number of patients (rectal cancer patients, mean predicted RSI, R vs. NR 0.32 vs. 0.46, p=0.03) (esophageal cancer patients, mean predicted RSI, R vs. NR 0.37 vs. 0.50, p=0.05).

We generated an ROC curve (Figure 2) using the predicted RSI to determine the sensitivity and specificity of the predictor. Using a threshold RSI of 0.46, the model has a sensitivity and specificity of 80 and 82% respectively, with a positive predictive value (PPV) of 86%. Although preliminary, these numbers are encouraging since the predictor is not developed to account for the radiosensitizing effect of chemotherapy and we expected the inclusion of chemotherapy to account for prediction inaccuracies.

The radiosensitivity predictive model is of prognostic value in HNC cancer

We further tested the model as a prognostic marker in locally-advanced HNC patients treated with definitive concurrent radiochemotherapy. The clinical details of this cohort have been previously published [19]. Briefly, the cohort included patients treated within Phase II and randomized Phase III trials at the Netherlands Cancer Institute (NKI). 94% of patients presented with T3 or T4 disease and all patients were treated with concurrent radiochemotherapy (cisplatin-based). Gene expression profiles for all patients were generated using the NKI array. Using the same algorithm developed in cell lines and tested in the rectal and esophageal cohorts, we generated radiosensitivity predictions for this dataset. Interestingly, the average RSI prediction was lower in this disease site when compared with rectal and esophagus (predicted RSI, HNC vs. esophagus vs. rectal 0.06 vs.0.43 vs.0.39). Although this could be partly a function of radiosensitivity differences between these diseases, it could also be due to platform differences (Affymetrix U133 Plus vs. NKI array). Interestingly, in spite of these differences, the RSI was still of prognostic value within the HNC dataset. The predicted radiosensitive group had an improved 2 year locoregional control (2yr LRC 86% vs. 61%, p=0.05), thus arguing that the model is capturing biological commonalities that determine tumor radiosensitivity across disease sites (Fig. 3).

Discussion

The development of in vitro diagnostics to predict response to therapeutic agents is a central goal of molecular medicine [1]. In this study we validate a robust systems-biology based multi-gene expression model of intrinsic tumor radiosensitivity in three independent datasets totaling 118 patients. Although previous studies have shown that radiosensitivity signatures were possible [15, 41, 42], this is the first time to our knowledge that a systems-biology based radiosensitivity model is validated in multiple independent clinical datasets. We show that RSI when analyzed as a continuous variable is correlated with pathological response in rectal and esophageal cancer patients treated with preoperative concurrent chemoradiation. Furthermore, the ROC analysis proposes a cut-point (RSI=0.46) where the test predictive accuracy is encouraging. The sensitivity, specificity and positive predictive value of the assay were 80%, 82% and 86% respectively. Importantly, we also show that RSI is of prognostic significance in a cohort of 92 patients with locally-advanced HNC. The applicability of the model in three different disease sites strongly suggests that the model captures commonalities that define radiosensitivity across disease sites. Therefore it is possible that the model might be generally applicable to other disease sites (e.g. lung, prostate, cervix cancer). However it should be

emphasized that no disease-specific conclusions should be made at this juncture given the small nature of two of the validating clinical cohorts (rectal, esophageal)

In the molecular medicine era, high-throughput technologies (e.g. microarrays, proteomics) have led to the identification of numerous molecular signatures of prognostic and/or predictive significance [10-12, 43]. However the initial enthusiasm that these signatures would lead to personalized medicine has been dampened by lack of robustness [44]. The robustness of the radiosensitivity model is supported by several lines of evidence. First, the algorithm was validated in three independent prospectively collected datasets in three different diseases. Second, the model was valid across different gene expression platforms. The model is originally developed on an Affymetrix HU-6800 platform but clinically validated in two different gene expression platforms (i.e. 1. Affymetrix U133-Plus for esophageal and rectal cohorts, 2. NKI cDNA array for HNC). This observation suggests the model can be transferred to a more practical clinical platform (i.e. RT-PCR/formalin-fixed tissue). Third, all patients in the validating clinical cohorts were treated with concurrent chemoradiation, since we were unable to obtain a dataset of patients treated with radiation alone. However the algorithm was based on cellular radiosensitivity. Thus, in spite of this potential source of inaccuracy, the model was still validated. Finally, the model showed both predictive and prognostic value.

False negatives (predicted radioresistant that responded) were the main inaccuracy when the model was dichotomized in the esophageal and rectal datasets. This population represented 60% of the misclassified cases in these cohorts. This inaccuracy may be due to the radiosensitization effect of chemotherapy. The proportion of individuals that are classified in this group (11.5%) is consistent with the observed improvement in clinical responses with concurrent chemotherapy over radiotherapy alone [45-47]. Therefore, this effect may be addressed by analyzing gene expression differences between responders and non-responders that share a predicted radioresistant phenotype.

The model in this study is designed to predict tumor radiosensitivity. Interestingly RSI was prognostic in the HNC dataset, suggesting that the biological factors that determine radiosensitivity are related to disease prognosis after treatment. This is not surprising since complete pathological response has been shown to have strong prognostic significance in several studies [18 48 49 17].

This model may play a central role in the individualization of therapy in radiation oncology. For example, the model may provide an opportunity to individualize radiation dose parameters based on intrinsic radiosensitivity. Since higher doses of RT are associated with higher toxicity rate [50], dose personalization would result in a therapeutic ratio benefit. There is also a role for identifying patients that are likely to be downstaged, particularly in rectal cancer. For example this knowledge might lead to better counseling of patients with low-lying rectal tumors

where sphincter-sparing surgery is being considered. In addition the model may provide a unique framework to understand the differences between responders and non-responders that share a predicted radioresistant phenotype. This may allow the accurate identification of patients that benefit from the addition of concurrent chemotherapy.

In conclusion we present evidence to support the clinical validity of a multi-gene expression model of intrinsic tumor radiosensitivity. To our knowledge this is the first systems biology-based radiosensitivity model to have validation in multiple independent datasets. The model is versatile and robust as demonstrated by both its predictive and prognostic ability in three different disease sites using two different gene expression microarray platforms. The data presented justifies further development and optimization of this technology in larger clinical populations.

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Legends

Figure 1. RSI is correlated with clinical response to concurrent radiochemotherapy in rectal and esophageal cancer patients. (A) The mean predicted RSI of responders is significantly lower than in non-responders in both clinical cohorts (esophageal: $p=0.05$, rectal: $p=0.03$). (B) Predicted RSI of each individual patient in the cohorts (combined: $p=0.001511$).

Figure 2. ROC curve using predicted RSI for radiosensitivity predictor. Using a threshold RSI of 0.4619592, the predictor has an 80% sensitivity and 82% specificity, with PPV of 86%. The estimated area under the curve (AUC) is 0.84.

Figure 3. RSI distinguishes clinical populations with different disease-related outcomes in a HNC cohort of 92 patients treated with definitive concurrent radiochemotherapy. Using the 25th percentile ($RSI < 0.023$), there is a superior 2 yr LRC in the predicted radiosensitive group (86% vs. 61%, $p=0.05$).

Figure 1

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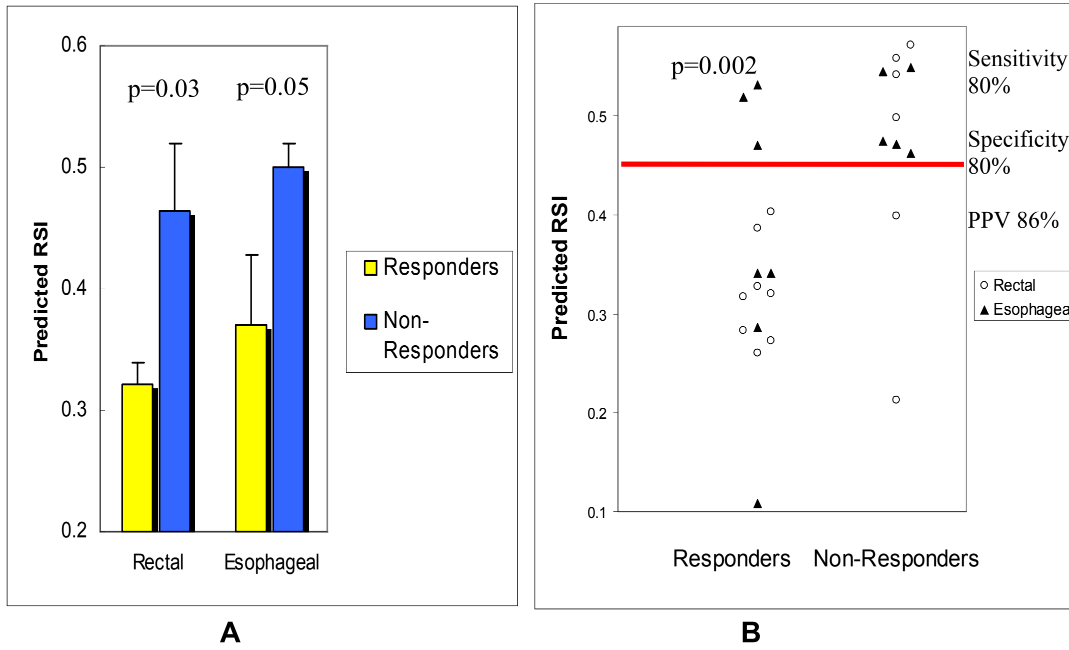

Figure 2

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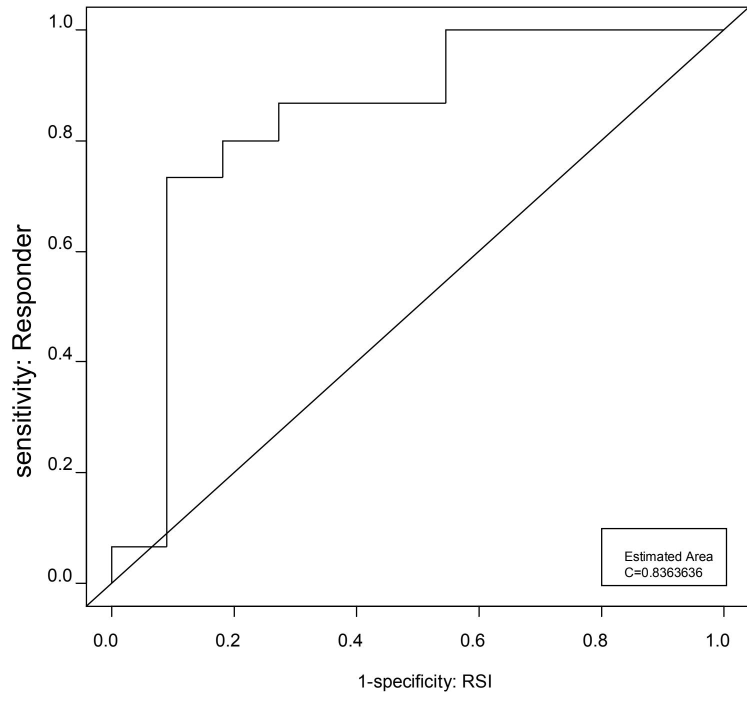

Figure 3
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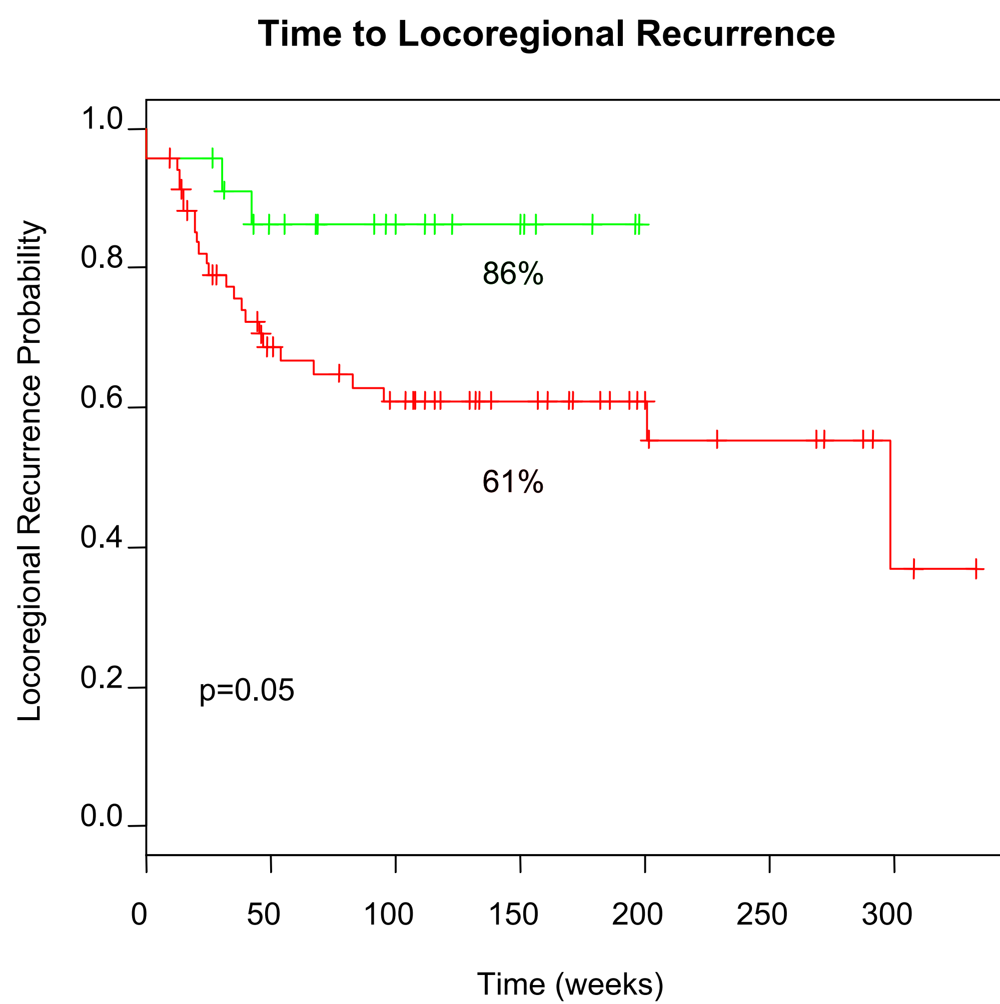

Table

Sex	Male	10	
	Female	4	
Age (y)	Mean	69.4	
	Median (range)	72	(50-90)
Chemotherapy Dose	0.25 mg/m^2/day	3	(21)
	0.4 mg/m2/day	5	(36)
	0.55 mg/m2/day	6	(43)
UltraSound Tumor Stage	T3	14	(100)
Pathological Tumor Stage	T0	2	(14.3)
	Tis	1	(7)
	T1	2	(14.3)
	T2	3	(21.4)
	T3	5	(36)
	T4	1	(7)
Downstaging	Yes	8	(57)
	No	6	(43)

Values are number (percentage) unless otherwise noted.

Table 1. Clinical Characteristics - Rectal Cancer Trial

Sex	Male	7	(58.3)
	Female	5	(41.7)
Age (y)	Mean	67.08	
	Median (range)	66	(51-80)
Chemotherapy Regimen	CDDP + 5-FU	4	(33)
	5-FU	2	(16.7)
	Carbo/Tax + 5-FU	1	(8.3)
	NA	5	(42)
Radiation Dose	45	1	(8.3)
	50.4	5	(42)
	54	2	(16.7)
	55.8	1	(8.3)
	61.2	1	(8.3)
	NA	2	(16.7)
Clinical Tumor Stage	T2N1	1	(8.3)
	T3N0	1	(8.3)
	T3N1	7	(58.4)
	T4N1	3	(25)
Pathological Tumor Stage	T0N0	4	(33.3)
	T0N1	1	(8.3)
	T1aN0	1	(8.3)
	T1N1	2	(16.7)
	T2bN1	1	(8.3)
	T2N1	1	(8.3)
	Progressive Dx	2	(16.7)
Downstaging	Yes	7	(58.3)
	No	5	(41.7)

Values are number (percentage) unless otherwise noted.

Table 2. Clinical Characteristics- Esophageal Trial

Gene Name	HU6800 Probeset	U133Plus Probeset	NKI Reporter
Androgen receptor	M23263_at	211110_s_at	324293
c-Jun	J04111_at	201466_s_at	329987
STAT1	AFFX-HUMISGF3A/M97935_MA_at	AFFX-HUMISGF3A/M97935_MA_at	308421
PKC	X06318_at	207957_s_at	322907
RelA (p65)	U33838_at	201783_s_at	326475
c-Abl	X16416_at	202123_s_at	304192
SUMO-1	U83117_at	208762_at	308596
CDK1 (p34)	U24153_at	205962_at	332859
HDAC1	D50405_at	201209_at	308690
IRF1	L05072_s_at	202531_at	310653

Table3. Radiation network hub genes. The probes used on each platform (Affymetrix HU6800, HGU133Plus2.0 and NKI cDNA arrays) are listed. Matches were identified via sequence similarity to the original HU6800 platform.