# Longitudinal Cytokine Expression during IMRT for Prostate Cancer and Acute Treatment Toxicity

Eva Christensen,[1,3] Melania Pintilie,[2,4] Kenneth R. Evans,[6] Michelle Lenarduzzi,[1] Cynthia Ménard,[1,3] Charles N. Catton,[1,3] Eleftherios P. Diamandis,[5,7,8] and Robert G. Bristow[1,3]

**Abstract**

**Purpose:** Proteomic profiling of patients undergoing intensity-modulated radiotherapy (IMRT) for prostate cancer can identify unique biomarkers that reflect acute toxicity in normal tissues. Our objectives were to measure inflammatory cytokine proteins during IMRT and assess the variability of individual proteomic signatures.

**Experimental Design:** Forty-two patients with intermediate-risk prostate cancer were recruited as follows: group 1, definitive IMRT (78 Gy in 39 fractions, $n = 22$), and group 2, IMRT postprostatectomy (66 Gy in 33 fractions, $n = 20$). Blood/urine samples were collected at baseline and weekly during IMRT. Acute toxicity was graded weekly during radiotherapy using CTC-AE v3.0 criteria. Multiplexed immunoassays were used to quantify cytokines including granulocyte macrophage colony-stimulating factor, IFN-γ, tumor necrosis factor-α, interleukin (IL)-1α, IL-2, IL6, IL-8, IL-10, and IL-12p70.

**Results:** We observed positive correlations between cytokine expression between serum and plasma, but not between serum/plasma and urine. The Mann-Whitney test showed a significant increase in IFN-γ and IL-6 during IMRT ($P = 0.0077$, 0.0035). Increasing IL-2 and IL-1 expression were associated with increased probability of acute gastrointestinal and genitourinary toxicity, respectively.

**Conclusions:** Determination of radiation-response signatures is feasible using multiplexed immunoassays and is a promising predictive early biomarker of toxicity outcomes. (Clin Cancer Res 2009;15(17):5576–83)

Cellular damage caused by ionizing radiation induces specific proteins involved in DNA repair, cell death, inflammation, and other pathophysiologic responses (1). The majority of biomarker studies in radiation oncology have focused on predicting tumor response and survival (2). Clinically, the acute toxicity of prostate cancer radiotherapy manifest as gastrointestinal and genitourinary symptoms based on validated scoring criteria, [e.g., Common Terminology Criteria for Adverse Events (CTC-AE); ref. 3]. Radiotherapy dose escalation using intensity-modulated radiotherapy (IMRT) is associated with improved biochemical tumor control, yet still has radiation-induced toxicity. Dose-dependent markers of acute normal toxicity could help predict individuals at increased risk of radiotherapy-related injury and help to maximize the therapeutic ratio for individual patients.

Rubin and colleagues were among the first to describe the role of cytokines (small glycoproteins involved in intercellular signaling) in mediating radiation toxicity (4). They showed in preclinical and clinical lung studies that levels of interleukin (IL)-1, transforming growth factor (TGF)-β, and tumor necrosis factor (TNF)-α were increased immediately after radiation exposure, and that chronically elevated TGF-β levels were associated with increased risk of pulmonary fibrosis. The link between radiation toxicity and cytokine expression is supported by studies showing that prolonged cytokine expression postradiotherapy is correlated to specific lung radiopathologies (2). Recently, Evans and colleagues assessed the utility of TGF-β as a predictor of radiation pneumonitis when combined with dosimetric and tumor parameters (5). They found that TGF-β ratios ≥1 were predictive of radiation pneumonitis in patients with 30 Gy delivered >30% of their lung volume. This agrees with other clinical studies (6, 7) that show that

**Authors' Affiliations:** [1]Radiation Medicine Program and [2]Department of Biostatistics, Princess Margaret Hospital, University Health Network, [3]Department of Radiation Oncology and Institute of Medical Science, [4]Dalla Lana School of Public Health Sciences, and [5]Department of Laboratory Medicine and Pathobiology, University of Toronto, [6]Ontario Cancer Biomarker Network, [7]Department of Pathology and Laboratory Medicine, Mount Sinai Hospital; and [8]Department of Clinical Biochemistry, University Health Network and Toronto Medical Laboratories, Toronto, Ontario, Canada
Received 2/1/09; revised 5/26/09; accepted 5/28/09; published OnlineFirst 8/25/09.

**Grant support:** National Cancer Institute of Canada, the Terry Fox Foundation and the Ontario Institute for Cancer Research (OICR-OCRN) to R.G. Bristow and K. Evans. E. Christensen was supported by a Matthews Family Scholarship in Radiation Therapy, a CIHR Strategic Training Fellowship in Radiation Therapy, and an Ontario Graduate Scholarship. R.G. Bristow is a Canadian Cancer Society Research Scientist.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

**Note:** Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

**Requests for reprints:** Robert G. Bristow, Radiation Medicine Program, Princess Margaret Hospital, 610 University Avenue, Toronto, Ontario, Canada M5G2M9, Phone: 416-946-2936; Fax: 416-946-4586; E-mail: rob.bristow@rmp.uhn.on.ca.



doi:10.1158/1078-0432.CCR-09-0245

### Translational Relevance

Developing biomarker profiles to assess normal tissue acute treatment response is an important step towards individualized radiation medicine. Cytokine expression is associated with radiation-related tissue damage and inflammation, and may be useful to triage radiation therapy patients if correlated to toxicity endpoints. In a highly defined prostate cancer patient cohort, we used a multiplexed immunoassay with the cytokines granulocyte macrophage colony-stimulating factor, IFN-γ, tumor necrosis factor-α, interleukin (IL)-1α, IL-2, IL6, IL-8, IL-10, and IL-12p70 to characterize longitudinal cytokine patterns during radiotherapy. We observed that IL-6, IFN-γ, IL-1, and IL-2 may be important cytokine markers of radiation response, specifically acute gastrointestinal and genitourinary toxicity. This work forms the basis for future prospective radiotherapy trials in which IL-6, IFN-γ, IL-1, and IL-2, if predictive of radiation toxicity, could ultimately alter patient management during radiotherapy.

increased TGF-β during radiotherapy predicts for radiation-induced pulmonary injury.

Ménard and colleagues (8) identified 23 protein fragments, including an IL-6 precursor, that were uniquely expressed in patients during radiotherapy compared with pretreatment controls. Although the majority of radiotherapy clinical studies looking for correlations between cytokines and treatment outcome have focused on measurements taken prior to and at the end of treatment, there are two notable longitudinal cytokine trials to mention. Allen and colleagues (9) found that longitudinal increases in IL-6, IL-8, vascular endothelial growth factor (VEGF), HGF, and GRO-1 during radiotherapy were significantly associated with decreased cause-specific survival in Head and Neck Squamous Cell Carcinoma (HNSCC). Similarly, Kovacs and colleagues (10) reported that expression patterns of IL-1α, M-CSF, and TGF-β follow cyclical waves in patients during conventional wide-field radiotherapy for prostate cancer, where waves of proinflammatory IL-1α and M-CSF preceded profibrotic TGF-β. In summary, although cytokine levels are up-regulated in response to radiation (8–11), the temporal pattern of these changes during radiotherapy has not been closely studied.

As cytokines may, in part, mediate radiation injury (2, 4, 12) it stands to reason that differential cytokine expression may serve as an indicator of cell and tissue toxicity during prostate cancer radiotherapy. We sought to determine temporal profiles of cytokine expression during radiotherapy for intermediate-risk prostate cancer patients treated with definitive and post-prostatectomy radiotherapy. The goals of this study were 3-fold: (*a*) to determine the feasibility and validity of longitudinal proteomics research within radiotherapy in terms of patient compliance, quality assurance, and sample handling variability; (*b*) to explore longitudinal serum cytokine patterns; and (*c*) to compare preradiotherapy cytokine signatures between the definitive and postprostatectomy cohorts to identify a prostate tissue–specific signature.

## Materials and Methods

***Study population.*** Forty-two patients with a diagnosis of prostate cancer and receiving either definitive IMRT of 78 Gy ($n$ = 22) or post-operative (adjuvant or salvage) IMRT of 66 to 70 Gy ($n$ = 20; 2 Gy per fraction) were enrolled in this pilot study. None of the patients received hormone therapy at any point during this study. Patients were recruited from December 2006 to December 2007. This study was approved by the University Health Network Research Ethics Board (UHN REB #06-0546-CE) and informed consent was obtained from all subjects prior to study enrolment.

Eligibility for this study was based on a diagnosis of histologically confirmed, locally confined prostate adenocarcinoma. The definitive radiotherapy group had intermediate-risk prostate cancer ($T_{1c\text{-}3C}$; Gleason score 6 or 7) and a maximum prostatic volume <80 cm (13). Patients in the postprostatectomy radiotherapy group ($pT_{2\text{-}4}$; Gleason score 6, 7, or 9) had a prostate-specific antigen (PSA) >0.05 ng/mL following surgery or positive surgical margins, and were prescribed IMRT. Patients were excluded if data regarding previous cancer therapy were unobtainable or if patients did not consent to serial blood and urine collections. All patients were planned and treated using image-guided IMRT. Definitive IMRT patients received a total dose of 78 Gy in 39 fractions, and post-prostatectomy patients received a total dose of 66 Gy in 33 fractions. IMRT planning to the prostate gland and definition of the postoperative group IMRT treatment volume have been previously described (14, 15). Quality assurance for image-guided radiotherapy was achieved by daily fiducial marker matching for definitive IMRT treatments and by bony anatomic matching for postoperative IMRT treatments.

***Patient blood and urine sampling and processing.*** Blood samples were obtained from patients at two pretreatment appointments (radiotherapy education and computed tomography simulation) and at every 5th fraction during IMRT. The first 13 patients had samples collected every 10th fraction. Patients had additional samples drawn on the last day of treatment. Ninety percent of patients had two baseline samples drawn preradiotherapy.

At every collection, a sample of peripheral blood was drawn into a vacutainer containing clot activator and gel for serum separation (BD Vacutainer). Samples were immediately placed on ice for transport to the laboratory. Time from sample collection to processing did not exceed 45 min. Provided that samples were kept on ice and centrifuged within 2 h, analysis of serum cytokine levels has previously been shown not to show any significant variability or artifacts *ex vivo* (16). Blood samples were centrifuged (3,000 $g$ × 10 min), and the platelet-free serum layers were separated from the blood and aliquoted into cryotubes that were coded and then frozen at -80°C until analysis.

***Grading radiation-induced toxicity.*** Patient-derived toxicity grading was prospectively collected using CTC-AE v3.0 (3, 17). Patients were asked to complete questionnaires concerning symptoms of rectal or bladder injury prior to and every week during radiotherapy as part of their standard care.

***Sample analysis.*** Reporting of cytokine measurements and analysis were completed using the National Cancer Institute and European Organization for Research and Treatment of Cancer recommendations for reporting tumor marker prognostic studies (REMARK; ref. 18). For IFN-γ, the average coefficient of variation (CV) of the assays was 14.6% ± 8.3 (range, 0.6-29.7). For IL-6, the average CV of the assays was 4.9 ± 4.3 (range, 0.2-16.3). Thus, the respective increases in IFN-γ and IL-6 reported across the entire cohort were within the average of the coefficient of variation (CV) of the assays, and in both cases the increase reported was greater than the maximum CV reported for the assays.

All analyses were carried out blind to patient and therapy factors. The expression of the cytokines granulocyte macrophage colony-stimulating factor, IFN-γ, TNF-α, IL-1β,IL-2, IL-6, IL-8, IL-10, and IL-12p70 were measured using a multiplexed immunoassay (Human Pro-inflammatory 9-Plex) from Meso Scale Discovery (MSD), LLC. In all cases, the assays were done according to the manufacturer's instructions. Cytokine concentration was quantified using a SECTOR Imager 2400 plate reader

**Table 1.** Patient and treatment-related demographics

| Factor | Definitive radiotherapy group | Postprostatectomy group |
|---|---|---|
| Age in y* | 72 ± 4 (60-82) | 62 ± 3 (51-74) |
| Clinical tumor stage[†] (*n*) | $T_{1c}$ (14)<br>$T_{2a}$ (6)<br>$T_{2b}$ (1)<br>$T_{3c}$ (1)<br>$T_3$ (2)<br>$T_{3a}$ (4)<br>$T_{3b}$ (2)<br>$T_4$ (2)<br>n/a (2)[‡] | $T_2$ (1)<br>$T_{2a}$ (2)<br>$T_{2b}$ (1)<br>$T_{2c}$ (4) |
| Gleason score (a + b)[†] | 3 + 3 (6)<br>3 + 4 (9)<br>4 + 3 (7)<br>4 + 5 (2)<br>5 + 4 (2)<br>n/a (1)[‡] | 3 + 3 (3)<br>3 + 4 (9)<br>4 + 3 (3) |
| Preradiotherapy PSA, ng/mL | 7.9 ± 1 (0.4-17.6) | 0.3 ± 0.6 (0-2.2) |
| Postradiotherapy PSA, ng/mL | 1.94 ± 1.29 (0.2-4.5) | 0.07 ± 0.17 (0-0.58) |
| Radiation dose | 78 Gy in 39 fractions | 66 Gy in 33 fractions |
| Mean PTV in $cm^{3*}$ | 163.64 ± 11.99 (99.18-353.96) | 368.71 ± 16.10 (242.42-506.40) |
| Mean minimum dose to PTV in cGy* | 7,231.94 ± 31.71 (6,862.50-7,380) | 5,142.14 ± 57.52 (4,237.60-5,374.40) |
| Mean maximum dose to PTV in cGy* | 8,172.07 ± 19.84 (7,929.10-8,343) | 7,129.43 ± 34.56 (6,862.40-7,648.50) |

*Data presented as the mean ± SE for 22 definitive radiotherapy alone and 20 postprostatectomy adjuvant radiotherapy alone patients entered into the study; distribution and/or range of data entries are provided in parentheses where applicable.
[†]Preoperative data provided for postprostatectomy patients.
[‡]Data not available.

(MSD) and preliminary analysis with MSD Discovery Workbench Software v2.0. Each assay was run against a standard curve with a full range predetermined for each cytokine and sample source. For each assay, a standard curve was generated for detection between 2.4 pg/mL to 10,000 pg/mL.

***Statistical analysis.*** All analyses were done using GraphPad Prism 5, SAS Version 9, and R software programs and with log-transformed data, unless otherwise stated. For all analyses, $P \leq 0.05$ was taken as significant.

The Mann-Whitney *U* test was used to compare baseline cytokine expression for definitive IMRT versus postprostatectomy IMRT patients. Spearman coefficients were used to assess correlations between cytokine expression in plasma, serum, and urine ($n = 12$, pilot analysis) and correlations between maximum cytokine expression and volume of irradiated tissue ($n = 42$). The Wilcoxon sign-rank test was used to determine the effect of radiation on cytokine expression using aggregated median cytokine levels at baseline versus during IMRT. The difference between baseline and during-radiotherapy cytokine levels was also tested using a mixed modeling approach where dose was disregarded to confirm the results of the signed-rank test. Cytokine levels were modeled over dose by applying mixed modeling using log-transformed protein values as outcome and dose as the explanatory variable.

Acute radiation toxicity was modeled as CTC-AE v3.0 grade 2 or 3 (defined as toxicity) versus 0 or 1 (defined as no toxicity). The cytokines, radiotherapy dose, and type of regimen were tested for their association with toxicity using a generalized estimation equation model.

## Results

The mean patient age in the definitive IMRT group was significantly older than the postoperative cohort (72 ± 4 years versus 62 ± 3years; $P < 0.0001$). The mean planning treatment volume (PTV) was 2.2-fold greater for the postprostatectomy group than the definitive IMRT group ($P < 0.0001$). The mean minimum and maximum doses to PTV were 1.4 and 1.1 times greater, respectively, for the definitive IMRT group compared with the postprostatectomy group ($P < 0.0001$ for both comparisons; see Table 1 for a summary of patient and treatment planning-related data). In the definitive IMRT group, PSA levels were significantly decreased immediately postradiotherapy at 6 to 8 weeks' follow-up versus preradiotherapy levels ($P = 0.020$). The change in PSA levels was not significant at the same time point for the postoperative group ($P = 0.30$).

***Baseline cytokine expression in definitive versus postoperative IMRT cohorts.*** The average time from sample collection to initial processing by centrifuge was 18.5 minutes (range, 9.3 to 40 minutes; Supplementary Table S1). In an analysis of 12 patients, there was a high correlation between the absolute levels of all cytokines tested in serum versus plasma (Supplementary Table S2). Because these samples were collected and analyzed separately, we conclude that the risk of artifact due to handling and processing was low. We subsequently focused on cytokine expression only in sera for the complete cohort of 42 patients. Patient compliance was excellent; of the 47 patients who originally consented, 89 % went on to complete the study. Regarding sample collection, 390 of 412 collections were successfully completed, which translates to a 95% success rate.

There were no significant correlations between any of the baseline cytokine levels and time from prostatectomy to start of radiotherapy for the postoperative cohort (Fig. 1). As such, we conclude that time from surgery to first fraction of radiotherapy treatment is not a significant confounding factor for cytokine expression in this treatment cohort. We also compared the baseline cytokine expression in the definitive IMRT group (intact prostate, $n = 22$) versus the postprostatectomy group (prostate removed, $n = 20$) to ascertain whether a

unique prostate-specific cytokine signature could be identified (i.e. cytokines that are elevated in the definitive IMRT group only; Supplementary Fig. S1). Median cytokine values ranged from 0.2 to 11.8 pg/mL (definitive) and from 0.6 to 13.7 pg/mL (postprostatectomy). None of the cytokines were uniquely elevated in the prostate-intact group. We conclude that there is no unique cytokine signature for prostate cancer for patients with an intact prostate within our cohort and our cytokine panel tested.

***Intrapatient variance of cytokine expression during radiotherapy.*** Figure 2 shows the ratios of cytokine variability within patients to the total variability calculated for the baseline and during-radiotherapy measurements with 95% confidence intervals. The ratio of the variance within patients to the total variance is reported as a measure of cytokine heterogeneity within the patient population. We observed that these ratios were similar at baseline and during IMRT for all cytokines tested.

Despite the variance being similar at baseline and during radiotherapy, we reasoned there could be differences between absolute expression levels. When the average cytokine levels from baseline and during radiotherapy were compared, we found that IFN-γ and IL-6 levels were significantly elevated in the treatment group over baseline ($P = 0.0077$ and 0.0035, respectively, $n = 42$; Fig. 3). Our models predicted that IFN-γ and IL-6 levels increase by 1.3- and 1.17-fold, respectively during radiotherapy as compared with baseline. When the treatment groups were analyzed separately, the difference in IFN-γ and IL-6 levels before versus during IMRT remained significant for the post-prostatectomy group, whereas for the definitive IMRT group the differences were not significant (Supplementary Fig. S2-S3).

We also calculated associations between cytokine expression and irradiated tissue volume given that both dose and volume irradiated are important variables in mediating radiation response. For IL-12 only, there was a borderline significant association between maximum cytokine level and volume of tissue irradiated for the definitive IMRT group ($P = 0.05$). For other cytokines there was no association between treatment volume and cytokine expression.

***Modeling time-dependent cytokine expression.*** We observed that IFN-γ and IL-6 levels were significantly associated with increasing radiotherapy dose over total treatment time. We investigated patterns of change in all cytokines relative to dose (Supplementary Fig. S4). IFN-γ levels were significantly associated with a linear model ($P = 0.009$) whereas IL-6 levels were consistent with our hypothesis and significantly associated with a quadratic function ($P = 0.001$). Based on this

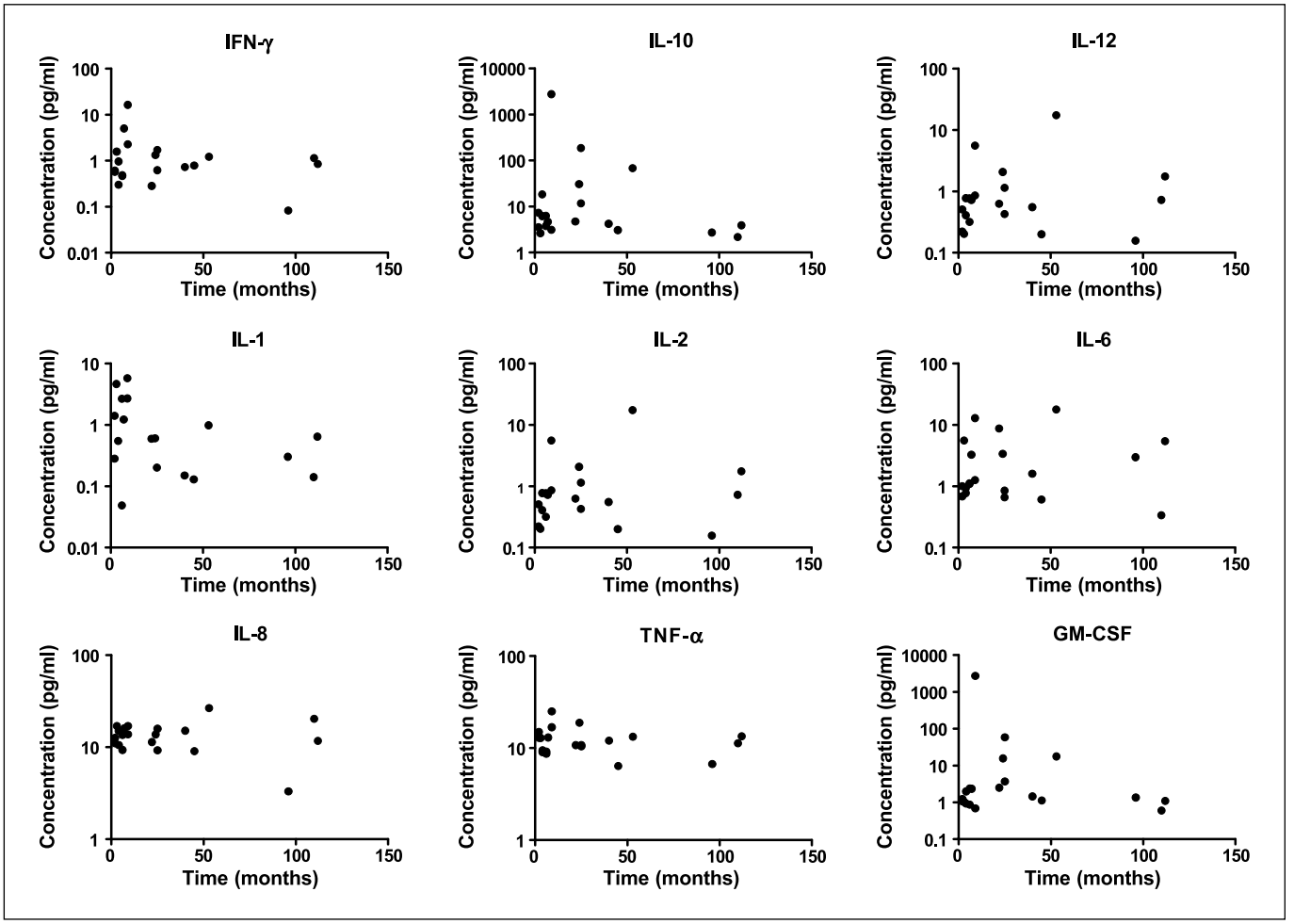


**Fig. 1.** Baseline cytokine levels as a function of time from radical prostatectomy to start of radiotherapy ($n = 42$). There is no correlation between this time interval and baseline cytokine levels (i.e., all $P > 0.05$).

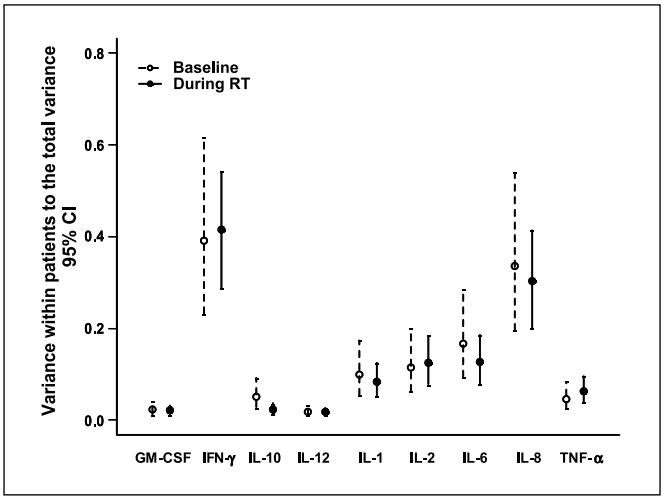


**Fig. 2.** Cytokine variance within patients as a measure of heterogeneity ($n = 42$, both IMRT regimens). We calculated the variance of each cytokine within patients and the total variance for each cytokine. The data points are the ratio of variance within to the total variance for each of the nine cytokines at baseline (*open circle*) or during IMRT (*black circle*) with 95% confidence intervals indicated. There is no significant difference between the ratios at baseline and during IMRT for any of the cytokines based on the confidence intervals shown (untransformed ratios normalized to total variance presented). However, the ratios are particularly large for IFN-γ and IL-8, which may indicate issues with data reproducibility in future experiments.

model, the IL-6 maximum was predicted to occur at 42 Gy. These data suggest that IFN-γ and IL-6 change as the dose of radiation increases.

***Associations with cytokine expression and patient-scored radiotherapy toxicity.*** We sought to determine if there was a relationship between cytokine expression and patient-reported gastrointestinal and genitourinary acute radiotherapy toxicity graded prospectively (summarized in Supplementary Table S3). The toxicity was modeled as grade 2/3 versus 0/1. Each of cytokine, radiotherapy dose, and the type of radiotherapy regimen was tested for association with toxicity using a generalized estimation equation model (19). In general, as the dose increased, the probability of genitourinary and gastrointestinal toxicity also increased. Increases in IL-2 and IL-1 levels over baseline were significantly associated with increased gastrointestinal and genitourinary toxicity respectively regardless of the IMRT regimen (Supplementary Fig. S5). The analysis of IL-6 suggested that the increase of IL-6 is associated with the higher probability for gastrointestinal toxicity but it did not reach statistical significance. With regard to gastrointestinal toxicity, the predicted probability for toxicity was 0.8% for definitive IMRT and 10.3% for postprostatectomy patients. Figure 4 summarizes maximum cytokine concentration versus maximum patient-scored genitourinary and gastrointestinal toxicity for cytokines of interest (IL-6, IFN-γ, IL-1, and IL-2) as identified by statistical modeling. The association between gastrointestinal and genitourinary toxicity with the cytokine levels is summarized in Fig. 5.

## Discussion

Preclinical and clinical studies have shown that radiotherapy induces cytokine responses that could play a major role in mediating radiation toxicity. In this study, IFN-γ and IL-6 were found to significantly increase during IMRT. Previous studies have shown that IL-6 is a radiation-inducible cytokine (20). For example, human lung fibroblasts respond to X-ray treatment with release of IL-6 (21). Recently, Ménard and colleagues identified an IL-6 precursor unique to radiation-exposed serum acquired from patients during radiotherapy (8). However, one drawback of their study was that it included a range of cancer diagnoses and radiotherapy treatment plans. In this study, these factors were closely controlled and we still observed elevated IL-6 levels during IMRT, supporting a bona fide IL-6 response to prostate radiotherapy.

There are limited data regarding the relationship between IFN-γ and radiation exposure, although IFN-γ is known to be involved in growth arrest in normal and tumor cells (22, 23). With regard to IL-1, we observed that its expression was associated with increased probability of genitourinary toxicity, although we were not able to establish a dose-response relationship as per Kovacs and colleagues (10). Previous work has shown that radiation induces IL-1, which acts as a radioprotectant of hematopoietic cells (24). Finally, the role of IL-2 in mediating radiation-induced toxicity is not well understood, although it is known to have multiple, sometimes opposing, functions during an inflammatory response, including stimulating the growth of T and B cells and the production of other cytokines (25).

The total volume of irradiated tissue usually is assumed to have an influence on the development of tissue injury (5, 20). Based on our models, IL-6 levels were predicted to be higher in the definitive IMRT group versus the postoperative for a given delivered dose. This was unexpected given that the postoperative group had a significantly larger irradiated volume (Table 1). This is in contrast with previous clinical research that has shown a correlation between radiation volume and toxicity in lung cancer patients. For example, Rodrigues and colleagues reviewed the literature and found that $V_{dose}$, defined as the volume of organ receiving a threshold dose (e.g., 25 Gy), was correlated with radiation pneumonitis in six previous studies (26). Evans and colleagues found that TGF-β1 is predictive for radiation pneumonitis in patients undergoing lung radiotherapy with high $V_{30}$ (>30% and TGF-β1 ratios ≥1 during radiotherapy; ref. 5). A lack of dose-volume effect for radiotherapy-induced cytokine expression for pelvis irradiation may be indicative of a low dose-volume threshold for cytokine induction whereby maximum cytokine expression is achieved over a narrow irradiation volume range. Although extensively studied, the exact relationship between radiotherapy-induced toxicity and pathology and dose-volume parameters is not straightforward. Morgan and Breitt discussed in a review that the degree of dyspnea observed in patients during lung radiotherapy is in fact out of proportion to the volume of lung irradiated (27), and Evans and colleagues also observed that gross tumor volume does not predict for TGF-β1 levels or radiation pneumonitis (5).

We observed that IFN-γ and IL-6 significantly increased during prostate IMRT, and we found a promising association between increase in IL-2 and IL-1 and acute gastrointestinal and genitourinary toxicity. Although statistically significant, this early data should be interpreted cautiously given the relatively small sample size and lack of toxicity events in the latter part of our analysis, but they deserve further study in a larger cohort.

One question arising from our results is why we did not observe the same patterns of cytokine expression for all patients during IMRT given that they received the same doses based on uniform dose volume histogram (DVH) parameters. Predictable patterns of cytokine expression have been observed previously in a number of murine studies. Rubin and colleagues studied cytokine induction in mice receiving thoracic irradiation of 5 and 12.5 Gy (4). At day 1, and 1, 2, 8, 16, and 24 weeks postradiation the animals were sacrificed and lung tissue was analyzed for cytokine RNA. They found that several cytokines were longitudinally elevated with consistent patterns between mice. For example, IL-1α was elevated at 2 weeks,

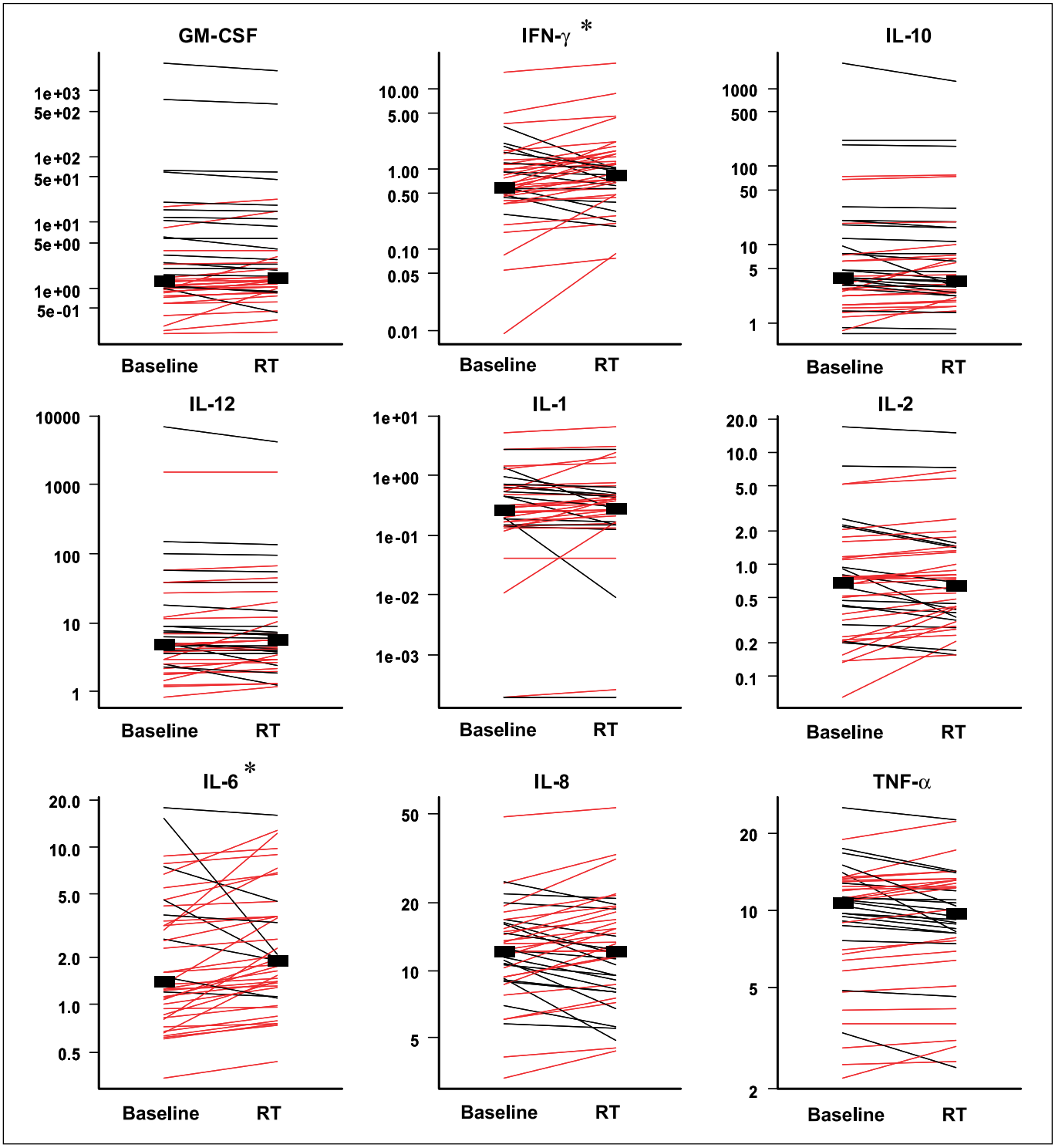


**Fig. 3.** Expression of individual cytokines for radiotherapy patients before and during radiotherapy. Cytokine levels for each patient were aggregated as baseline versus IMRT. Wilcoxon sign-rank analysis was used to identify cytokines for which there was a significant overall change between baseline and IMRT. IL-6 and IFN-γ levels are significantly increased during IMRT versus baseline ($n = 42$; $^*P < 0.05$). Solid boxes, median values.

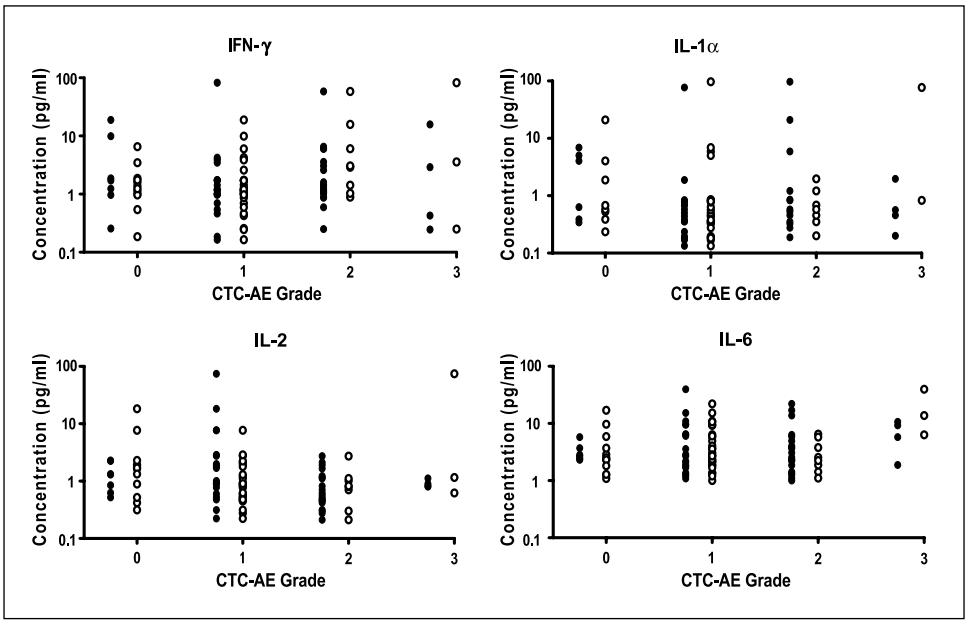


**Fig. 4.** Relationship between cytokine expression and acute toxicity during IMRT. Maximum cytokine expression (IFN-γ, IL-1α, IL-2, IL-6) as a function of maximum toxicity grade for $n = 42$ patients. Open circle, genitourinary toxicity; closed circle, gastrointestinal toxicity.

returned to baseline, increased at 8 weeks, and remained elevated until 26 weeks when lung fibrosis appeared. Not only did this study show the early and persistent elevation of cytokine production following pulmonary irradiation, but the researchers were also able to determine consistent patterns of expression for multiple cytokines of interest. In contrast, we observed a high degree of interpatient variability in cytokine expression and were only able to model patterns for IL-6 and IFN-γ. That we were not able to determine IMRT patterns of response of all cytokines tested can likely be attributed to the complexity of our patient sample. Despite uniform prostate cancer diagnoses and IMRT treatment plans, our patients had a range of comorbidities and lifestyle factors that may have significantly affected their cytokine profiles and which we were not able to account for in our analysis.

We also investigated pretreatment baseline cytokine expression in a definitive IMRT group (prostate intact) in comparison with the unique control of postprostatectomy patients (with prostates removed) to establish whether a unique prostate-specific signature could be identified. In preclinical work, IL-6 has been shown to act as a growth factor for androgen-independent prostate cancer cells, in addition to activating signal transduction cascades that in turn stimulate androgen receptor activity in prostate cancer cells (28). In clinical studies, Shariat and colleagues found that plasma IL-6 levels are significantly elevated in patients with prostate cancer metastatic to bone (29). They also observed that preoperative plasma IL-6 levels predicted for biochemical progression and lymph node metastases in men following radical prostatectomy (30). Further studies are clearly required to attribute the increased expression of any cytokine in prostate cancer patients as a biomarker of malignancy and tumor response to radiotherapy. The latter will require long-term (e.g. >5 years) coordinated follow-up in these cohorts.

The statistical analysis presented here was intended to be exploratory and to guide future, larger trials. Future studies in larger groups of patients are warranted to determine the time course of serum cytokine changes after radiation exposure and how these relate to normal tissue radiation toxicity with greater certainty. An example would be to compare cytokine expression measurements in patients receiving hypofractionated versus standard definitive fractionation schedules as in the

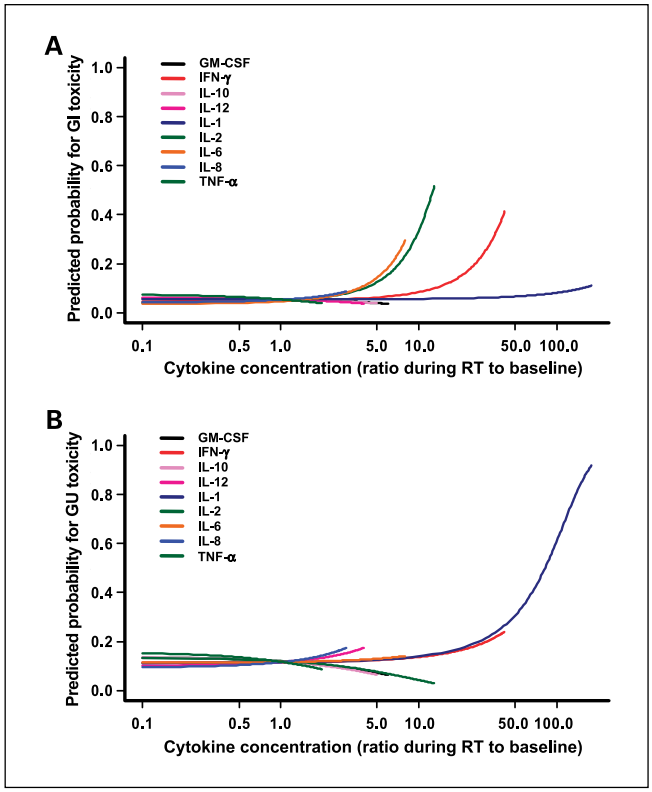


**Fig. 5.** Modeled associations between predicted probability of toxicity and change in cytokine over baseline for all cytokines. *A*, based on our dataset, IL-6, IL-2, and IFN-γ are proteins of interest for predicting gastrointestinal toxicity as all of these cytokines are associated with increased gastrointestinal toxicity. *B*, for genitourinary toxicity, IL-1 and IFN-γ are cytokines of interest given that changes in these cytokines over baseline are associated with increased genitourinary toxicity during IMRT.

PROFIT IMRT trial (31) that compares the efficacy of 60 Gy in 20 fractions with 78 Gy in 39 fractions for the treatment of localized prostate cancer.

In conclusion, proteomics research is feasible and valid based on our study methodology. Future work should focus on time-dose protein patterns in a larger cohort of patients given that our preliminary statistical analysis shows a promising relationship between IMRT toxicity and cytokine expression.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

## Acknowledgments

We thank Ms. Grace Abankwah, Debbie Tsuji, and Linda Purushuttam for their assistance consenting patients and phlebotomy service.

## References


1. Faulhaber O, Bristow RG. Basis of cell kill following clinical radiotherapy. In: Sluyser M, editor. Application of apoptosis to cancer treatment. Springer; 2005, p. 293–320.
2. Okunieff P, Chen Y, Maguire DJ, Huser AK. Molecular markers of radiation-related normal tissue toxicity. Cancer Metastasis Rev 2008;27: 363–74.
3. National Cancer Instititute Cancer Therapy Evaluation Program. Common Terminology Criteria for Adverse Events. 2006, version 3.0.
4. Rubin P, Johnston CJ, Williams JP, McDonald S, Finkelstein JN. A perpetual cascade of cytokines postirradiation leads to pulmonary fibrosis. Int J Radiat Oncol Biol Phys 1995;33:99–109.
5. Evans ES, Kocak Z, Zhou SM, et al. Does transforming growth factor-beta1 predict for radiation-induced pneumonitis in patients treated for lung cancer? Cytokine 2006;35:186–92.
6. Anscher MS, Marks LB, Shafman TD, et al. Risk of long-term complications after TFG-β1-guided very-high-dose thoracic radiotherapy. Int J Radiat Oncol Biol Phys 2003;56:988–95.
7. De JK, Seppenwoolde Y, Kampinga HH, Boersma LJ, Belderbos JS, Lebesque JV. Significance of plasma transforming growth factor-β levels in radiotherapy for non-small-cell lung cancer. Int J Radiat Oncol Biol Phys 2004;58:1378–87.
8. Menard C, Johann D, Lowenthal M, et al. Discovering clinical biomarkers of ionizing radiation exposure with serum proteomic analysis. Cancer Res 2006;66:1844–50.
9. Allen C, Duffy S, Teknos T, et al. Nuclear factor-κB-related serum factors as longitudinal biomarkers of response and survival in advanced oropharyngeal carcinoma. Clin Cancer Res 2007;13: 3182–90.
10. Kovacs CJ, Daly BM, Evans MJ, et al. Cytokine profiles in patients receiving wide-field + prostate boost radiotherapy (xRT) for adenocarcinoma of the prostate. Cytokine 2003;23:151–63.
11. Chen Y, Hyrien O, Williams J, Okunieff P, Smudzin T, Rubin P. Interleukin (IL)-1A and IL-6: applications to the predictive diagnostic testing of radiation pneumonitis. Int J Radiat Oncol Biol Phys 2005;62:260–6.
12. Denham JW, Hauer-Jensen M. The radiotherapeutic injury-a complex "wound". Radiother Oncol 2002;63:129–45.
13. Lukka H. Prostate cancer: risk categories and role of hormones and radiotherapy. Can J Urol 2002;9 Suppl 1:26–9.
14. Wiltshire KL, Brock KK, Haider MA, et al. Anatomic boundaries of the clinical target volume (prostate bed) after radical prostatectomy. Int J Radiat Oncol Biol Phys 2007;69:1090–9.
15. Huang SH, Catton C, Jezioranski J, Bayley A, Rose S, Rosewall T. The effect of changing technique, dose, and PTV margin on therapeutic ratio during prostate radiotherapy. Int J Radiat Oncol Biol Phys 2008;71:1057–64.
16. Christensen E, Evans KR, Menard C, Pintilie M, Bristow RG. Practical approaches to proteomic biomarkers within prostate cancer radiotherapy trials. Cancer Metastasis Rev 2008;27:375–85.
17. van der Laan HP, van den Bergh A, Schilstra C, Vlasman R, Meertens H, Langendijk JA. Grading-system-dependent volume effects for late radiation-induced rectal toxicity after curative radiotherapy for prostate cancer. Int J Radiat Oncol Biol Phys 2008;70:1138–45.
18. National Cancer Institute PftAoCCT. REporting recommendations for tumor MARKer prognostic studies (REMARK). 2007.
19. Hanley JA, Negassa A, Edwardes MD, Forrester JE. Statistical analysis of correlated data using generalized estimating equations: an orientation. Am J Epidemiol 2003;157:364–75.
20. Hall EJ. Clinical response in normal tissue. Radiobiology for the radiologist. 5th ed. New York: Lippincott, Williams & Wilkins; 2000, p. 339–60.
21. Brach MA, Gruss HJ, Kaisho T, Asano Y, Hirano T, Herrmann F. Ionizing radiation induces expression of interleukin 6 by human fibroblasts involving activation of nuclear factor-kappa B. J Biol Chem 1993;268:8466–72.
22. Ekmekcioglu S, Mumm JB, Udtha M, Chada S, Grimm EA. Killing of human melanoma cells induced by activation of class I interferon-regulated signaling pathways via MDA-7/IL-24. Cytokine 2008;43:34–44.
23. Kim KS, Kang KW, Seu YB, Baek SH, Kim JR. Interferon-γ induces cellular senescence through p53-dependent DNA damage signaling in human endothelial cells. Mech Ageing Dev 2009;130:179–88.
24. Neta R. Modulation with cytokines of radiation injury: suggested mechanisms of action. Environ Health Perspect 1997;105 Suppl 6: 1463–5.
25. Hoyer KK, Dooms H, Barron L, Abbas AK. Interleukin-2 in the development and control of inflammatory disease. Immunol Rev 2008; 226:19–28.
26. Rodrigues G, Lock M, D'Souza D, Yu E, Van DJ. Prediction of radiation pneumonitis by dose-volume histogram parameters in lung cancer – a systematic review. Radiother Oncol 2004;71: 127–38.
27. Morgan GW, Breit SN. Radiation and the lung: a reevaluation of the mechanisms mediating pulmonary injury. Int J Radiat Oncol Biol Phys 1995;31:361–9.
28. Smith PC, Hobisch A, Lin DL, Culig Z, Keller ET. Interleukin-6 and prostate cancer progression. Cytokine Growth Factor Rev 2001;12:33–40.
29. Shariat SF, Andrews B, Kattan MW, Kim J, Wheeler TM, Slawin KM. Plasma levels of interleukin-6 and its soluble receptor are associated with prostate cancer progression and metastasis. Urology 2001;58:1008–15.
30. Shariat SF, Kattan MW, Traxel E, et al. Association of pre- and postoperative plasma levels of transforming growth factor beta(1) and interleukin 6 and its soluble receptor with prostate cancer progression. Clin Cancer Res 2004;10: 1992–9.
31. U.S.National Institutes of Health. PROFIT-Prostate Fractionated Irradiation Trial. 2009.