Assessing patient characteristics and radiation-induced non-targeted effects in vivo for high dose-rate (HDR) brachytherapy

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Abstract

Purpose: To test whether blood, urine, and tissue based colony-forming assays are a useful clinical detection tool for assessing fractionated treatment responses and non-targeted radiation effects in bystander cells.

Materials and methods: To assess patients’ responses to radiation treatments, blood serum, urine, and an esophagus explant-based in vivo colony-forming assay were used from oesophageal carcinoma patients. These patients underwent three fractions of high dose rate (HDR) intraluminal brachytherapy (ILBT).

Results: Human keratinocyte reporters exposed to blood sera taken after the third fraction of brachytherapy had a significant increase in cloning efficiency compared to baseline samples (p < 0.001). Such results may suggest an induced radiosensitivity response in bystander cells. The data also revealed a clear inverse dose-rate effect during late treatment fractions for the blood sera data only. Patient characteristics such as gender had no statistically significant effect (p > 0.05). Large variability was observed among the patients’ tissue samples, these colony-forming assays showed no significant changes throughout fractionated brachytherapy (p > 0.05).

Conclusion: Large inter-patient variability was found in the urine and tissue based assays, so these techniques were discontinued. However, the simple blood-based assay had much less variability. This technique may have future applications as a biological dosimeter to predict treatment outcome and assess non-targeted radiation effects.

Keywords: Bystander effect, high dose-rate (HDR) brachytherapy, esophageal carcinoma, urine, blood, esophageal explants

Introduction

High dose-rate intraluminal brachytherapy (HDR-ILBT) has established itself as an effective treatment modality for patients diagnosed with advanced stages of esophageal cancer (Sur et al. 2002). Brachytherapy enables high doses of radiation to be delivered to the tumor to improve the patients’ dysphagia scores, quality of life (Berry et al. 1989), has the advantage of providing conveniently fast outpatient procedures (Sur et al. 1998). A remote afterloading HDR unit is used for delivering high doses of gamma radiation, from an Iridium-192 (Ir¹⁹²) source, to the tumor site (Sur et al. 2002). A rapid drop-off of dose from the treatment site to surrounding normal tissues results in a very small risk of injury to nearby normal tissues (Yoshioka et al. 2013). Fractionated HDR-ILBT has shown to significantly improve dysphagia-free survival and longevity in comparison to other palliative modalities (Sur et al. 1998).

Dose to normal tissues typically restricts treatment planning protocols for radiotherapy modalities, as these are limited by normal tissues tolerance doses (Mothersill et al. 2004a). However, in vitro research has documented the biological implications of bystander factors being released into non-irradiated cells which has been shown in the literature to trigger a cell death response (Mothersill et al. 2004b). Consequently, non-targeted radiation effects can ultimately affect treatment planning protocols, as there is a possibility of much larger out-of-field effects in normal tissues than initially expected (Butterworth et al. 2013). Other work has suggested that radiation-induced bystander effects (RIBE) may provide insight into understanding the efficacy of radiotherapy, as bystander factors may enhance tumor cell killing (Boyd et al. 2008, Prise and O’Sullivan 2009, Butterworth et al. 2013). Currently, it is not fully understood whether the release of bystander signals into healthy surrounding tissues, near radiation fields, leads to unwanted damage in normal cells (Brenner et al. 2000, Hall and Wu 2003, Mothersill and Seymour 2006, Boyd et al. 2008). Therefore we and others have extended the investigation of non-targeted radiation effects from an in vitro experimental approach (Mothersill and Seymour 1997, Prise et al. 1998, Lyng et al. 2000, Seymour and Mothersill, 2000) to the whole organism by using animal
(Morgan 2003, Chai and Hei 2008, Koturbash et al. 2008) and human models (Seymour and Mothersill, 2000, Mothersill et al. 2002, Marozik et al. 2007, Chai and Hei 2008).

For the past several years, non-targeted radiation effects such as clastogenic effects (Seymour and Mothersill 2006, Howe et al. 2009), RIBE (Emerit et al. 1995, Mothersill and Seymour 1997, Ryan et al. 2009) and adaptive responses have been well documented. Radiation-induced clastogenic effects are found in atomic bomb survivors (Pant and Kamada 1977), humans undergoing radiotherapy (Seymour and Mothersill 2006), and in the blood serum collected from Chernobyl liquidators (Marozik et al. 2007).

One of the earlier studies observing clastogenic effects was published by Goh and Sumner (1968); the study evaluated chromosomal aberrations in cultivated leukocytes treated with blood plasma taken from patients that underwent total body irradiation. The findings showed that blood plasma exposed to radiation increased the number of chromosome breaks in leukocytes compared to unirradiated samples. Similar clastogenic effects have also been reported by other investigators in the literature with humans (Hollowell and Littlefield 1968, Pant and Kamada 1977, Emerit et al. 1994, 1995, 1997) and animals (Faguet et al. 1984) following radiation exposure.

Earlier work focused on exploring the variability inherent in human urothelial tissue explants and their ability to express bystander signals in reporter cells (Mothersill et al. 2002). Signal production was found to be sex-specific and had a dependence on whether the participants had no existing malignancies. A gender discrepancy was observed, tissue samples harvested from female participants resulted in a higher reduction in cloning efficiency compared to males. Other researchers used a rodent model to assess non-targeted radiation effects within non-irradiated spleens following cranial radiation exposure (Koturbash et al. 2008). These authors found male mice to be more susceptible to bystander effects in comparison to females.

Another very important phenomenon associated with non-targeted radiation effects is induced radioresistance responses. This cell protective effect is not unique to radiation alone, rather it has been observed with acute hypoxia-induced stimuli within analogous systems and many different cell types (Michiels 2004). The induction of radioresistance responses were found in areas of high natural background radiation in Ramsar compared to control populations from regions of low background radiation (Mohammadi et al. 2006). Lymphocytes were extracted and exposed to 4 Gy of gamma radiation, and individuals residing in high natural radiation background areas had significantly higher DNA damage and repair than control groups (Mohammadi et al. 2006). Other investigators assessing fractionated X-ray treatments, found enhanced clonogenic survival following subsequent treatments in radiosensitive clones of human colorectal tumor cell lines (Qutob et al. 2006).

In the present study, non-targeted radiation effects were assessed with an in vivo-based assay for blood, urine, and esophageal biopsy samples taken before and after a fractionated brachytherapy regime. The primary motivation of this study was to explore radiation-induced bystander effects (RIBE) in blood, urine, and biopsy samples taken from esophageal cancer patients undergoing fractionated HDR-ILBT. Secondary objectives were to assess whether blood and urine samples pre-exposed to one treatment fraction of brachytherapy induces radioresistance, by stimulating an increase in reporter cells survival, during subsequent exposure to brachytherapy. Additionally, certain patient characteristics were assessed to determine whether these variables are influencing cell communicating signals that ultimately affects cell cloning capabilities.

Based upon previous in vitro studies (Boyden and Raaphorst 1999, Maguire et al. 2007), it is hypothesized that fractionated treatments will induce a cell communicating protective response in reporter cells exposed to patient samples taken following each fraction of brachytherapy. This work will contribute to the limited data available and further our understanding of non-targeted radiation effects in brachytherapy at therapeutic doses.

**Materials and methods**

**Sample design**

Blood, urine and biopsy samples were obtained from patients diagnosed with either esophageal adenocarcinoma (EA) or squamous cell carcinoma (SCC) undergoing HDR-ILBT between March 2011 and February 2012. The majority of patients were males diagnosed with esophageal adenocarcinoma (EA). Roughly 54% of the patients had stage III cancer and 26.7% had stage IV cancer, refer to Table I for patient characteristics and demographics. This research was carried out according to the Declaration of Helsinki with informed consent obtained from all participants, and ethics approval was obtained from the Hamilton Health Sciences

| Table I. Patient demographics and clinical characteristics. | No. of patients (n = 15) |
| --- | --- |
| Patient characteristics | |
| Mean age in years (SD) | 69.3 (10.6) |
| Gender n (%) | |
| Male | 11 (73.3%) |
| Female | 4 (26.7%) |
| Type of cancer n (%) | |
| SCC | 3 (20.0%) |
| EA | 12 (80.0%) |
| Cancer staging n (%) | |
| Stage II | 1 (6.7%) |
| Stage III | 8 (53.3%) |
| Stage IV | 4 (26.7%) |
| Not reported | 2 (13.3%) |
| Tumor location n (%) | |
| GEJ | 11 (73.3%) |
| Mid/Upper esophagus | 1 (6.7%) |
| Above GEJ | 3 (20.0%) |
| Metastases n (%) | |
| Yes | 5 (33.3%) |
| No | 8 (53.3%) |
| Not determined | 1 (6.7%) |
| Smoking status n (%) | |
| Current | 2 (13.3%) |
| Former | 12 (80.0%) |
| Never | 1 (6.7%) |
| Mean dose-rate (Gy/h) (SD) | |
| Fraction 1 | 59.4 (25.2) |
| Fraction 2 | 68.2 (23.4) |
| Fraction 3 | 65.4 (20.0) |
Faculty of Health Sciences (HHS/FHS) research ethics board (REB # 06-193). In the present study, 24 patients were eligible for recruitment, however, only 11 men and four women, with a mean age of 69 years (range age, 57–90 years) participated in the study. Out of the 15 patients, two patients discontinued from the study after the first fraction of HDR-ILBT, one patient refused to undergo fraction 2 and 3 of brachytherapy, and one patient was deemed ineligible to participate after the first fraction of treatment by the attending physician. All patients received 600 cGy per HDR fraction prescribed 1 cm from the source axis to the esophageal planning volume with a remote afterloading HDR unit (Varisource HDR, Varian International, USA) administering high doses of gamma radiation, by using a Ir¹⁹² source. The length of the treatment field is determined at the time of endoscopy which occurred right before the catheter is set in place on the day of brachytherapy. Appropriate margins were set based on clinical visual determination of the tumor where a 2 cm treatment margin was added proximal and distal to the tumor. The dose-rate ranged anywhere between 33.1 and 109.0 Gy/h. Further details on eligibility criterion has been described in a small pilot study published elsewhere (Pinho et al. 2012).

Sample collection

Blood and urine samples were collected at the start and end of each fraction of HDR-ILBT. Tissue specimens were biopsied from the tumor-free mucosa layer of the esophagus, proximal to the tumor site. A biopsy puncture technique was used to extract tissue specimens ranging in size from 1–2 mm². Biopsies of the esophagus were obtained prior to the first fraction of HDR-ILBT (baseline sample) and immediately following the final fraction of treatment (test sample).

Urine samples were placed in a 70 ml sterile container (Sarstedt, Montreal, QC, Canada) and peripheral blood samples in a 10 cc red lid serum Vacutainer containing no additive (BD Vacutainers, Fisher Scientific, Ottawa, ON, Canada). The samples were placed in a collection holder on ice immediately following extraction to maintain the integrity of the sample. For serum extraction, blood samples were centrifuged at 2000 rpm for 10 min and the serum was aliquoted into 5 ml sterile polypolyylene tubes (Sarstedt, Montreal, QC, Canada). The serum was extracted from blood samples within 2 h of being collected from patients in the clinical trial laboratory at the Juravinski Cancer Centre (JCC). Biopsy samples were collected and transported in 15 ml sterile polypolyylene tubes containing RPMI medium with a final concentration of 200 U/ml penicillin and 200 μg/ml streptomycin solution, 15 mM HEPES buffer, 1 μg/ml of Fungizone, 50 μg/ml of Nystatin, 0.5 μg/ml of hydrocortisone, and 2 mM of L-glutamine solution. Culture medium and supplements were obtained from Invitrogen Burlington Ontario. All samples were transported on ice to our research laboratory at McMaster University and were processed within 8 h of being collected.

Cell line

Human keratinocytes HPV-G cultures (Pirisi et al. 1988) were used as a reporter to determine whether bystander signals were being generated, following HDR-ILBT, in blood serum, urine and esophageal samples. The human keratinocyte reporter model has been widely accepted in a number of labs to have a well-characterized and stable bystander response over a large range of doses (Lyng et al. 2006, Mothersill et al. 2001, Ryan et al. 2008, Ahmad et al. 2013). The complete growth medium used for routine maintenance and colony-forming assays was RPMI-1640 with 10% foetal bovine serum (FBS) (Invitrogen, Burlington, ON, Canada), 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco, Burlington, ON, Canada), 2 mM L-Glutamine (Gibco, Burlington, ON, Canada), 0.5 μg/ml of hydrocortisone (Sigma-Aldrich, Oakville, ON, Canada), and 15 mM of Heps. All experiments were performed in a class II biosafety cabinet at McMaster University. Routine subculturing was performed on cell stocks reaching 80–100% confluency by using a 1:1 solution of 0.25% trypsin and 1 mM EDTA at 37°C for 8 min. Cell stocks were grown in 75 cm² flasks (T-75) filled with 30 ml of supplemented growth medium. Cell stocks and colony-forming experiments were incubated at 37°C and 5% carbon dioxide in air.

Tissue explants

Prior to fraction 1 and immediately following fraction 3, biopsies were taken as described above. Tissue dissections were not needed since three biopsies were taken at fraction 1 and 3. Each piece of tissue collected was approximately 1–2 mm³ in size and these samples were aseptically plated in the center of 25 cm² flasks (T-25) filled with 4 ml of supplemented growth medium. The complete growth medium for clonogenic assays was similar to the tissue sample collection medium except for the exclusion of antimycotics and a final concentration of 100 U/ml penicillin and 100 μg/ml streptomycin solution (Gibco, Burlington, ON, Canada) was used. The esophageal explants were placed in the incubator at 37°C in 5% carbon dioxide in air for 48 h.

Clonogenic assay

Explant conditioned medium

For esophageal explants, HPV-G reporters were set up at a density of 500 cells per T-25 flask containing 4 ml of culture medium. Explant conditioned medium (ECM) was generated by incubating the esophageal explants in culture medium for 48 h as described above. After 48 h, a standard medium transfer was performed where the ECM was filtered with a 0.22 μm Nalgene filter (VWR Burlington, Ontario, Canada) and placed onto reporter cultures. Following medium transfers, reporters were grown in an incubator at 37°C with 5% carbon dioxide in air for 10–14 days. Once viable colonies had formed, the cells were stained with 20% carbol fuchsin (VWR, Burlington, Ontario, Canada) and colonies with ≥ 50 cells were scored. Biopsies taken at the start of fraction 1 prior to the patient undergoing HDR-ILBT were used as controls. Biopsies taken immediately following irradiation were treatment samples.

Blood serum

Seymour and Mothersill (2006) developed a blood serum in vivo colony-forming assay to assess human subject responses to radiation treatment. In the present
study, this technique was utilized on esophageal cancer patients undergoing fractionated brachytherapy. On the evening prior to patient treatments, human keratinocyte reporters were seeded at 300 cells per T-25 flask containing 5 ml of RPMI containing a final concentration of 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco, Burlington, ON, Canada), 2 mM L-Glutamine (Gibco, Burlington, ON, Canada), 0.5 μg/ml of hydrocortisone (Sigma-Aldrich, Oakville, ON, Canada), and 15 mM of Hepes. The FBS typically used in growth medium was substituted with 10% blood serum (0.5 ml per 5 ml of culture medium) collected before and after treatments. Treatments were performed early morning and blood serum was extracted at the JCC. All samples were then transported to McMaster University. The serum was added to the medium and then transferred to reporter flasks. Similar to explants clonogenics, reporters were incubated at 37°C with 5% CO₂ in air for 10–14 days, and then stained and colonies with ≥ 50 cells were scored.

**Urine samples**
The in vivo colony-forming assay with urine samples was developed by Pinho et al. (2012) and preliminary data were published in Pinho et al. (2012). These clonogenic assays were performed alongside biopsy and blood sample experiments. Human keratinocyte reporters were seeded with 700 cells in T-25 flasks in 5 ml of RPMI culture medium. Urine samples were diluted 10-fold and added to the flasks. 1 ml of diluted pre- and post-treatment urine samples were added to flasks. Control flasks with an additional 1 ml volume of diluted medium (diluted with sterile distilled water) was set up to ensure 1 ml volume of diluted urine with medium did not affect the colony-forming ability of reporters. Reporter cells were incubated at 37°C with 5% CO₂ in air for 10–14 days, and then stained and colonies with ≥ 50 cells were scored.

**Statistical analysis**
All reporter flasks were set up in triplicate for each sample and at every fraction of brachytherapy. Data presented in this paper display three measurements per patient at each treatment fraction. A Shapiro-Wilk normality test and Levene’s tests found that the data violated the normality and equal variances conditions required for a parametric statistical analysis. When assessing whether blood and urine samples repeatedly taken from patients at various time-points throughout brachytherapy had a distinct treatment effect, an non-parametric Friedman’s test with a post hoc Wilcoxon signed rank test were performed. The p-values were adjusted with Bonferroni corrections to eliminate the chance of committing type I errors. When before and after treatment groups were compared for patient characteristics or tissue explant clonogenic assays, significance was determined by performing separate Wilcoxon signed rank tests. For the urine based colony-forming assay, there was a limited number of female patients able to give a sample. As a result, patients’ cancer staging characteristics were analyzed only. The relationship between cell survival and dose-rate was assessed using a Spearman’s correlation on the blood and urine samples. Statistically significant correlations were analyzed further with a linear regression analysis. A complete analysis on all 15 patients was not feasible for a number of reasons including patients leaving from the study, patients unable to give urine samples, and logistical difficulties associated with sample collection. All p-values less than 0.05 were considered statistically significant.

**Results**

**Blood based colony-forming assay**
Figure 1 shows the relationship between cloning efficiency and dose-rate administered at each fraction of brachytherapy. Patients undergoing fraction 2 and 3 of brachytherapy demonstrated a significant moderate positive relationship between cloning efficiency and dose-rate ($p < 0.05$), whereas, fraction 1 had no such relationship. The association between cloning efficiency and dose-rate were assessed further with a linear regression analysis for fractions 2 and 3 of brachytherapy. A positive trend between cloning efficiency and dose-rate was observed for fractions 2 ($p < 0.05$) and fractions 3 ($p < 0.001**$). This model indicates that 29.7% and 36.3% of the total variation with the cloning ability of non-irradiated keratinocytes can be explained by the dose-rate for fractions 2 and 3, respectively (Figure 1). The data is showing bystander reporters exposed to blood sera taken from cancer patients under-
going high dose-rate brachytherapy had a clear inverse dose-rate effect during late treatment fractions. The dose-rate variability observed across each of the fractions of brachytherapy can be explained in part by the patients’ tumor size, but most likely the dose-rate differences between fractions is related closely with the decay parameters (i.e., source decay and source renewal), refer to Supplementary data in Figure 1, available online at http://informahealthcare.com/abs/doi/10.3109/09553002.2015.1068458.

When running the repeated measures analyses, brachytherapy revealed a statistically significant increase in cloning efficiency for HPV-G reporters treated with blood sera amongst the treatment fractions \((p < 0.001^*)\), refer to Figure 2. Although when examining each treatment fraction, it can be seen that samples taken before brachytherapy compared to post-treatment samples revealed no statistical changes in cloning efficiencies \((p > 0.05)\). Rather a significant increase in the colony-forming ability of non-irradiated reporters was observed at the later part of brachytherapy. For instance, the final fraction of brachytherapy had a statistically significant increase in cloning efficiency by 12.60% relative to baseline samples \((p < 0.001^*)\). Whereas post-treatment samples at fractions 1 and 2 had an insignificant increase in cloning efficiency by 7.22% \((p = 0.705)\) and 8.65% \((p = 0.210)\) compared to baseline samples, respectively. To eliminate the chance of committing type I errors with Wilcoxon multiple pairwise comparisons when assessing treatment effects at various points in time, each \(p\)-value was adjusted with Bonferroni corrections refer to (Supplementary data in Table 1, available online at http://informahealthcare.com/abs/doi/10.3109/09553002.2015.1068458).

When exploring the outcome of cloning efficiency in respect to gender for samples taken before and after treatment, these patient characteristics were found to have no significant influence on the growth of non-irradiated cells \((p > 0.05)\), refer to Figure 3a. In contrast to gender differences, cancer staging showed a significant increase in cloning efficiencies for patients clinically diagnosed with stage III at fraction 1 of treatment and stage IV at fraction 2 of brachytherapy, as shown in Figure 3b.

**Urine based colony-forming assay**

The relationship between cell survival and dose-rate was also assessed for urine samples. For patients undergoing fraction 1, 2, and 3 of brachytherapy, there was no relationship between cell survival (%) and dose-rate (Figure 4).

Similar to the blood sample data, urine samples had a statistical difference in cell survival throughout the course of
Instead of the effect occurring in the post-treatment samples at the final fraction of treatment, samples taken before the third fraction of brachytherapy had a significant increase in cell survival compared to post-treatment samples after the first fraction of brachytherapy ($p_{H11021} < 0.001^*$. In Figure 5, it can also be seen that samples taken after fractions 1 and 2 had insignificant changes in cell survival compared to baseline samples. Similar to the blood sample results, these findings are suggesting that late treatment fractions of brachytherapy are inducing a radioresistance response in non-irradiated cells. Furthermore, cancer staging had no influence on the growth of non-irradiated cells ($p_{H11022} < 0.05$), however, the data reveals large variability for this endpoint (Figure 6).

A significant increase in the survival of non-irradiated reporters was also observed at the later part of brachytherapy ($p < 0.05^*$), as shown in Figure 5 and Supplementary data in Table 2. available online at http://informahealthcare.com/abs/doi/10.3109/09553002.2015.1068458. Instead of the effect occurring in the post-treatment samples at the final fraction of treatment, samples taken before the third fraction of brachytherapy had a significant increase in cell survival compared to post-treatment samples after the first fraction of brachytherapy ($p < 0.001^*$). In Figure 5, it can also be seen that samples taken after fractions 1 and 2 had insignificant changes in cell survival compared to baseline samples. Similar to the blood sample results, these findings are suggesting that late treatment fractions of brachytherapy are inducing a radioresistance response in non-irradiated cells. Furthermore, cancer staging had no influence on the growth of non-irradiated cells ($p > 0.05$), however, the data reveals large variability for this endpoint (Figure 6).

Figure 4. The relationship between cell survival (%) for HPV-G reporters and dose-rate of brachytherapy for urine samples are illustrated for fractions 1, 2, and 3. The sample size consisted of 11 patients. Outlined above are three measurements set-up per patient for each fraction of brachytherapy ($n = 33$). There were no statistically significant relationships found for fraction 1, 2, and 3.

Figure 5. Illustrated in the graph is the cell survival (%) of HPV-G reporters after exposure to diluted urine samples before and after fractionated brachytherapy. Lettering indicates similarities and significance between the treatment groups. Eight patients had triplicate flask set-up for each fraction of brachytherapy. All values are mean $\pm$ SEM for $n = 24$.

Figure 6. Cancer staging were also assessed across each treatment group to explore whether any changes in clonogenic survival (%) was dependent on certain patient characteristics. There were 8 and 4 patients clinically diagnosed with cancer stage III and IV, respectively. All values are mean $\pm$ SEM for $n = 24$ and $n = 12$ for cancer stage III and IV, respectively.

Figure 7. Shown above is the HPV-G reporters’ clonogenic survival (%) after being exposed to explant conditioned medium with samples taken before the first fraction and immediately after the final fraction of brachytherapy. Six patients had triplicate flask set-up for each treatment. All values are mean $\pm$ SEM of $n = 18$. 
Tissue explant based colony-forming assay

For the biopsy samples, tissues taken at baseline had a lower survival by 6.00% compared to samples taken immediately following the final treatment of brachytherapy ($p > 0.05$). These results reveal no indication of tissue explants generating bystander signals following brachytherapy.

Discussion

The primary objective of this study is to determine whether blood, urine, and tissue explant-based colony forming assays can be used to trace levels of bystander or protective signals being generated following brachytherapy treatments. A few patient characteristics were assessed to determine whether these factors might be influencing cell communicating signals and affecting the growth of non-irradiated cells.

Although the data is limited, the findings may suggest that cancer patients undergoing fractionated brachytherapy induced a radioresistance response for cells or tissues in close proximity to the irradiated tumor volume after undergoing treatment 3 compared to baseline samples. Such a response was observed in bystander cells exposed to blood sera and urine samples taken from esophageal cancer patients irradiated in vivo. Urine sample results had a similar trend as the blood serum data, although, these samples did not have a significant increase in cell survival when taken immediately after fraction 3. Instead, urine samples taken before fraction 3 had a significant increase in the reporters cell survival compared to post-treatment samples taken after the first fraction of brachytherapy.

In the literature, there is a considerable amount of research on cellular radioresistance responses performed in vitro (Thomas et al. 2013), once cells have been exposed to small acute doses or low acute dose-rates, initiating protective responses or enhanced repair processes. However, the induced radioresistance response is commonly triggered with doses below 1 Gy and dose-rates ranging from 0.18–2.43 Gy/min (Thomas et al. 2013). In the present study, irradiations occurred in vivo with a prescribed dose to the esophageal lumen of 600 cGy per HDR fraction and the average dose-rates per fraction were $>50$ Gy/h. Our findings are suggesting an induced radioresistance response after subsequent treatment fractions. However, a characteristic of this phenomenon is that low doses below 1 Gy are required during in vitro irradiations. One possible explanation for the effect being triggered late into brachytherapy regimes with substantially higher doses and dose-rates, would be that circulating blood cells flowing through the tumor volume may have been directly irradiated with significantly lower doses of gamma radiation than the tumor itself. However, there is also a possibility that blood cells flowing nearby the tumor spend significantly less time in the radiation field and may have not been directly irradiated, rather the effect may be a systemic immune response (Mothersill and Seymour 2004). The response observed in the non-irradiated cells, incubated with supernatants of blood serum during the final fraction of brachytherapy, are presumably initiated as a result of neighboring cells receiving comparable doses to in vitro radiation studies.

Side-effects in radiotherapy regimes are primarily attributed to different patients having inherently unique radiosensitivities (Twardella and Chang-Claude 2002). One of the first promising studies assessing the RIBE and cancer patients’ intrinsic radiosensitivities from blood samples was published by Howe et al. (2009). In this study, it was shown that lymphocyte cultures, taken from colorectal cancer patients, had a significant increase in radiosensitivity and its ability to produce bystander signals compared to cancer-free controls. Other studies detected bystander and radioprotective factors in the blood serum of Chernobyl accident survivors (Marozik et al. 2007) and cancer patients undergoing various fractions of radiotherapy (Seymour and Mothersill 2006), respectively.

Bystander signalling has been suggested to be associated with the activation of macrophages in mice (Lorimore et al. 2001). Recently researchers have shown that radiation stimulates the innate immune function (Manda et al. 2012, Multhoff and Radons 2012, Rödel et al. 2012, Mothersill 2013). With such high doses being prescribed at each fraction, further investigation with a macrophage Superoxide Dismutase (SOD) assay revealing innate immune function may be beneficial to test in future work (Johnston et al. 1978, Fukasawa et al. 1988). The superoxide anion ($O_{2}^-)$ is a short-lived free radical that plays an essential role in immune responses (Johnston et al. 1978). Such a radical is commonly released from macrophages. Macrophages collected from blood and cultured using regular cell culture techniques would be one way of investigating whether this response is a systematic immune response. A more mechanistic approach to elucidate other cellular activities would be to assess reactive oxygen species (ROS) activity in the bystander cells by using the 2’,7’-Dichlorofluorescein (DCF) fluorescent probe (O’Dowd et al. 2006). This marker for ROS can be loaded into the human keratinocytes cells after exposure to medium supplemented with 10% blood serum taken from cancer patients undergoing brachytherapy. Past work has found that increased levels of fluorescence has been highly correlated with higher levels of ROS in bystander cells (O’Dowd et al. 2006).

From our experiments, the urine-based colony-forming assays showed substantial inter-patient variability relative to the blood based assay results. The urine-based assay had proved to be unreliable and was deemed unsuitable for further clinical work due to the large variation observed amongst patients and treatment fractions. However, the blood based assay had much less variability and revealed interesting findings that provides further insight on the previously published work (Seymour and Mothersill 2006, Marozik et al. 2007). Unlike previous in vitro work resulting in a lower ability to produce bystander signal(s) when tissue samples were harvested from males with a pre-existing malignancy (Mothersill et al. 2002), the present clinical study had no such influence on signal production for gender. Furthermore cancer staging had no observable influence on the growth of non-irradiated reporters in cancer patients undergoing brachytherapy for the urine samples, but there was a significant effect observed for cancer staging for blood serum data only. These inconsistent results are most likely attributed to the small sample size and would need to be assessed further with a larger sample.
The data also revealed a clear statistically significant inverse dose-rate response in bystander cells exposed to blood serum harvested immediately following brachytherapy at fractions 2 and 3. However, the urine samples had no such effect observed following fraction 2 and 3 treatments. An earlier study by Mitchell et al. (1979), observed an inverse dose-rate effect during in vitro radiations of HeLa cells exposed to dose-rates ranging from 0.37–1.54 Gy/h (Mitchell et al. 1979). These authors found that HeLa cells had an increase in cell death for lower dose-rates than higher ones. At certain dose-rates, HeLa cells progress through the cell cycle and become blocked in the radiosensitive G2 phase at lower dose-rates, resulting in enhanced cell killing.

One of the most problematic issues that should be addressed in this paper were the loss of valuable biopsy samples, due to patient contamination. Patient contaminations were attributed to opportunistic yeast infections such as Candida albicans, which are common in immunocompromised cancer patients as explained in Delsing et al. (2012). Due to the scarcity of data available on such infections for esophageal carcinoma patients (Chiou et al. 2000), these issues were originally overlooked at the start of the clinical study. Throughout the remainder of the sample collection, antifungics (fungizone and nystatin) were supplemented in the collection medium prior to commencing the tissue explant colony-forming assays as a preventive measure. It is our recommendation for other investigators to either check for yeast infections or to supplement fungizone and nystatin for a short duration of time to avoid similar problems. As it stands, the tissue explant colony-forming assays led to inconclusive data and even more unanswered questions. Further investigations are warranted to assess the radiosensitivity and non-targeted radiations effects in nearby esophagus samples.

Another limitation associated with this study was the small sample size and the substantial inter-patient variation observed from one treatment fraction to the next. The limited sample size was attributed to difficulties with consenting patients for biopsy samples, as well as for all stages of treatment, and 3 weeks follow-ups (unreported data). The number of subjects required to achieve statistical power was conducted with a power analysis using G*Power software (Faul et al. 2007) with a power (1-β) set at 0.95 and α = 0.05. Future work would need to have a sample size of 84 in order to determine whether the different treatment fractions changes in cell survival have reached statistical significance at the 0.05 level. In retrospect, a much simpler study design focusing on only one treatment fraction and blood serum samples alone would be more appropriate since esophageal cancer typically presents at advance stages where the diseases progression are quite poor (Sur et al. 1998).

In conclusion, this simple blood-based assay may have future implications as a clinical detection tool used to predict treatment outcome based upon certain patient characteristics, such as gender, cancer staging, and metastatic status. From these preliminary findings, a follow-up study with a larger sample size, including unirradiated cancer-free controls, and a thorough analysis of patient characteristics may shed light on whether this technique may be appropriate as a predictive assay for assessing radiation side-effects or treatment outcome. A follow-up study has been undertaken with a target sample size of 115 cancer patients and 15 healthy patients with a power (1-β) set at 0.95 and statistical significance level set to 0.05. This work will provide further insight on whether non-targeted radiation effects have relevance in HDR brachytherapy.

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Supplementary material available online

Supplementary Tables 1 and 2 and Figure 1 available online at http://informahealthcare.com/abs/doi/10.3109/09553002.2015.1068458.