Gene Expression Studies for the Development of Particle Therapy

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Abstract
Proton therapy for cancer is now in widespread use, and facilities for carbon ion therapy are showing great promise, but a more complete understanding of the mechanisms underlying particle radiation therapy is still needed in order to optimize treatment. Studies of gene expression, especially those using whole genome techniques, can provide insight into many of the questions still remaining, from the molecular mechanisms involved to predicting patient outcome. This review will summarize gene expression studies of response to proton and carbon ion beams, as well as high-energy protons and high-z high-energy particles with relevance to particle therapy. In general, most such studies find that, in comparison with x-ray or gamma-ray exposure, particle irradiation increases both the number of genes responding and the magnitude of the response. Patterns of gene expression have suggested impacts on specific pathways of relevance to radiation therapy, such as enhancement or suppression of tumor progression or metastasis. However, even within the relatively small number of studies done to date there is no clear consensus of response, suggesting influence by multiple parameters, such as particle type, particle energy, and tumor type. Systematic gene expression studies can help to address these issues, and promoting a culture of data sharing will expedite the process, benefiting investigators across the radiation therapy field.

Keywords
gene expression; carbon ion; proton; high-z high-energy; particle therapy

Introduction
Proton therapy has become increasingly widespread for cancer treatment, and there is strong interest in the development of treatment using heavier ions, with the current focus on carbon ions. As of March 2018, the Particle Therapy Co-Operative Group (https://ptcog.ch) reported 68 proton therapy facilities worldwide and 11 carbon ion facilities already operating in Asia and Europe, with more under development. So far, results from these facilities have shown great promise, particularly for some hard-to-treat cancers, such as pancreatic cancer [1], with further trials ongoing. Despite the interest in ion therapy, many...
questions remain, and a better understanding of the mechanisms involved could help to
guide development. More detailed knowledge is needed in several broad areas to optimize
treatment response in particle therapy. Many of the gaps in current knowledge center around
an understanding of the relative biological effectiveness (RBE) for tumor cell killing and
normal tissue damage as a function of linear energy transfer (LET).

Some factors that affect these responses include tumor or tissue type, dose and dose rate,
fractionation schemes, tumor hypoxia, individual variation in sensitivity, as well as immune
and other abscopal responses [2]. Such studies may also lead to development of combined
therapy using more than 1 ion species, which might be beneficial in some circumstances [3].
Developing a knowledge base in these areas should also help to address the fundamental
question of whether carbon is, in fact, the optimal heavier-than-proton ion for cancer
treatment. Since lighter ions could be produced more cost effectively than carbon, it is
important to understand if they could provide the same or possibly superior therapeutic
benefit before greater investment in carbon ion infrastructure.

A broad range of preclinical studies and experimental techniques will be necessary to obtain
the knowledge needed to guide the future development of particle therapy. Gene expression
profiling represents one such approach that has emerged over the past decade as a powerful
means of addressing a variety of basic and clinical questions, including those in radiation
therapy. Gene expression profiles can be used to predict sensitivity of tumors to radiation [4,
5] and the response of patients to radiation therapy [6–8], to identify “druggable” targets for
modification of radiation response [9], or to identify signal transduction pathways or
biological functions responding to different types of radiation [10–12]. In addition,
development of deconvolution algorithms now also allows expression profiles from mixed
cell types to be applied to the study of tumor progression [13, 14] or tumor-infiltrating
immune cells [15]. Emerging systems biology approaches that combine gene expression
profiling with information from other biological “levels,” such as oncogene status, protein
expression, or metabolomic profiling, can also accelerate discovery and provide mechanistic
insight, as well as helping to develop biomarkers to guide radiation therapy [4, 16–18]. As
no single experimental technique or model can perfectly address all the nuances of complex
clinical questions, one of the strengths of a systems-level approach is the integration of data
not only from different technical platforms and assays, but also from different biological
models.

Despite the ever-increasing insight that may be gained from gene expression data, these
approaches have not been fully exploited for understanding the mechanisms underlying
particle radiation therapy, especially for particles heavier than protons. Gene expression
studies could help to address key questions in particle radiation therapy, including the impact
of dose, dose rate, fractionation, hypoxia, and tumor type on the mechanisms of cell killing
and immune activation as a function of LET. Such studies could also be exploited to identify
druggable targets for optimizing combined therapy, and to develop predictive tests for
individual sensitivity to particle therapy, both in terms of tumor response and normal tissue
toxicity. This review will summarize the gene expression studies using therapeutic proton
and carbon ion beams as well as some studies of high-energy protons and high-z high-
energy particles that have come out of the space radiation research field.
Responses in Normal Tissue

Minimizing damage to normal tissue is a goal in all radiation therapy, and owing to the physics of energy deposition, this is one of the perceived strengths of particle therapy. The photons used in traditional radiation therapy deposit energy throughout the target tissue, resulting in a relatively high entrance dose and a residual exit dose to the normal tissue. In contrast, most of the energy of protons or charged particles is deposited near the end of the track, at the Bragg peak, resulting in much less exposure to normal tissue when treating deep tumors [19]. Preclinical studies exposing cells or small animals to these beams can be challenging to conduct and interpret; however, owing to the different LET distributions within a clinical spread-out Bragg Peak (SOBP) in human patients and in the much thinner experimental samples. The energy of clinical beams, or the high-energy particle beams available at the NASA Space Radiation Laboratory at the Brookhaven National Laboratory, can be lowered with the use of physical absorbers, but this results in degraded beams contaminated by neutrons and breakup products [20], which may in turn affect the induced gene expression responses. One study exposed human fibroblast cultures at 3 positions along a proton beam profile, the entrance, mid-SOBP, and the SOBP distal edge (where the LET peaks), and reported that position within the beam had little effect on the induced expression changes of the 17 genes studied [21]. While this is encouraging, a fuller understanding of the LET dependence of gene expression effects is still needed.

Many of the gene expression studies related to particle radiation therapy have focused on the response of nontransformed cells or normal tissues, generally comparing the results of particle irradiation with those of a reference photon radiation (x rays or gamma rays). Among such studies, most report a greater effect of protons or heavier particles on gene expression, both in terms of the number of genes responding and the magnitude of the response. This trend was reported both for the significantly responding genes in whole genome studies [22, 23], and for selected gene sets representing extracellular matrix and adhesion molecules [24], stem cell differentiation [25], or oxidative stress [26]. In contrast with these findings, one study found that genes related to inflammation showed a lower magnitude of induction in primary fibroblast cultures after proton exposure than after gamma-ray exposure [21].

Consistent with the involvement of more genes in the gene expression response to particle irradiation compared with photons, several studies report responses unique to specific radiation qualities. For instance, the spleens of mice exposed to gamma rays showed a proapoptotic profile that was not observed after exposure to protons (1 GeV), which seemed to elicit a functionally more diverse gene expression response [27]. Ding et al [28] compared the dose- and time-dependent gene expression response of human bronchial epithelial cells to gamma rays with their response to silicon or iron ions (both 1 GeV/n) and found that the greatest variation in gene expression was due to radiation type. They identified a 73-gene signature that could predict the type of radiation to which a sample had been exposed with high accuracy.

Different patterns of gene expression consistent with different observed cellular or physiological responses have also been reported following high and low doses of protons.
The gastrointestinal tract of mice exposed to 1- or 2-Gy protons (250 MeV) showed an apoptotic gene expression signature not seen after a 0.1-Gy exposure [29]. In another study, exposure of a human 3-dimensional skin model to 2.5-Gy protons (4.5 MeV) altered the expression of genes associated with loss of structural integrity, terminal differentiation, and p53 response, while the response to 0.1 Gy was dominated by genes with functions in tissue repair and recovery [30].

Gene expression is also known to vary as a function of the rate of exposure, although there are few data available in this area for either particles or photons. This is an area of high relevance to particle therapy owing to the trend toward active beam scanning, which delivers instantaneous dose rates of hundreds of Gy/min in very rapid pulses. Such dose rates raise issues of local cellular oxygen depletion and altered distribution of free radical species, which could potentially alter biological responses [3]. One study comparing the response of genes involved in DNA damage and p53 signaling to protons delivered by passive or active beam scanning in human lung epithelial cells found a more than 6-fold increase in the number of genes responding to the same dose delivered by active beam scanning [31]. More studies are needed in this area, and also studying the impact of hypoxia within tumors.

**Individual Sensitivity**

Some studies have also begun to probe interindividual variation in normal tissue damage following charged particle therapy. For instance, in a study using 3 strains of mice with different patterns of disease development following lung irradiation, Moritake et al [32] showed different gene expression patterns following gamma-ray or carbon ion (290 MeV/n) irradiation that may contribute to susceptibility to early hemorrhagic pneumonitis after carbon ion exposure. Another study compared the expression response of a panel of inflammation-associated genes in primary fibroblast cultures from 30 radiosensitive or radioresistant patients, and found that cells from sensitive patients had the greatest upregulation of inflammation genes with less dependence on radiation type (gamma rays or protons) [21].

While these studies have focused on gene expression profiles in the target tissue or cell type, there is also great interest in the development of profiles to predict radiation toxicity or therapy outcome from a peripheral blood biopsy. These approaches are being developed for photon radiation therapy, and include both gene expression in peripheral blood cells either before or after irradiation [33–38], and profiling of plasma miRNA [39, 40]. Such approaches are particularly appealing, as they would allow minimally invasive testing to help shape the course of treatment, or to monitor progress. Studies of the response in blood to proton and charged particle exposures have so far largely focused on discrimination of these exposures from photon exposure, or on other basic aspects of exposure marker development and biodosimetry [41–46], but blood-based patient screening approaches could also be developed for ion therapy applications.
Responses in Cancer Cells or Tumors

Many studies have also examined the gene expression response in tumors or cancer cells exposed to particle irradiation. A study of the effects of protons (35 MeV) on the human colon carcinoma cell line HT29 found that proton exposure resulted in the downregulation of genes involved in integrin signaling and cell adhesion by 24 hours after irradiation, suggestive of inhibition of metastatic potential by proton treatment [47]. Similar results were reported after proton (3 MeV) exposure of the human A549 non-small cell lung carcinoma cell line. Here protons produced a much greater gene expression response than gamma rays, as well as reducing cell adhesion and migration, and reducing expression of stem cell markers [48]. Exposure to carbon ions (290 MeV/n) also suppressed the metastatic potential of 2 lung cancer cell lines and was associated with an induced gene expression signature distinct from that induced by x-rays [49]. Gene expression in response to carbon ions in this study again reflected significant downregulation of pathways associated with metastasis. The human prostate cancer cell line PC3 also showed a similar response to carbon ions (75 MeV/n), with greater downregulation of genes involved in cell cycle and cell motility when compared to the x-ray response [50]. The reverse was found in the Caco-2 colon cancer cell line, however, where motility-related genes were more strongly downregulated by x-ray treatment compared with carbon ions, suggesting a complex relationship of LET-dependent response to tumor or cell type [51].

Furthermore, a study using immortalized and oncogenically progressed human epithelial lung cells reported a strong prosurvival and cellular transformation signature among genes induced by iron or silicon ions (1 GeV/n) within the same time frame, with less effect induced by x-rays, suggesting that exposure to some particles may be more efficient at inducing tumor progression when initiated cells are present [12]. Using a mouse model of colon cancer initiation and progression, Kim et al [52] also found that exposure to protons (50 MeV) was more efficient at tumor initiation than x-rays, and this was associated with a greater induction of senescence-associated inflammatory response genes, and Plat, a gene involved in tumor invasiveness, when assayed 50 to 100 days after exposure. Protons (5 MeV) have also been reported to enhance the expression of a gene signature associated with epithelial to mesenchymal transition [53], a step in tumor progression.

It also appears that the impact of ion exposure on tumor progression may differ with age. Studies using a lung tumor model in mice identified a number of key gene expression changes that appeared to be associated with decreased tumor progression when young animals were irradiated with iron ions (1 GeV) [54]. Dramatic differences were also found in age-dependent gene expression following exposure to protons (1 GeV). Proton exposure resulted in a gene expression profile in spleen supporting cell cycle progression and immunosuppression in irradiated adolescent, but not aged, hosts [55]. Tumors grown in aged mice also showed significant transcriptome alterations linked to Tgfβ1 and Tgfβ2, supporting a further reduction of the older hosts’ already diminished capacity to support tumor advancement [56]. The expression of inflammation-associated and proangiogenic genes was also found to be suppressed in several human cell lines after exposure to protons (1 GeV), while the same genes were upregulated after exposure to gamma rays [57].
Tamaki et al [58] reported no differences in the expression profiles of lung metastases that occurred in a mouse model after treatment of primary tumors with carbon ions or gamma rays, suggesting that despite potential alterations in propensity for progression and metastasis, both radiation qualities may nonetheless drive a similar pathway of tumor development. The different conclusions reached by the studies discussed above may reflect differences in cell lines, tumor type, times of measurement, or other factors, and further studies on the impact of particle irradiation on tumor promotion, metastasis, or the initiation of second cancers are needed to more fully understand the balance of risks involved.

The effects of combining carbon ion treatment with chemotherapy have also been investigated at the gene expression level. Carbon ion irradiation combined with gemcitabine was reported to be more effective than x-rays plus gemcitabine or carbon ions alone at reducing expression of cancer stem cell markers in stemlike cells sorted from 2 pancreatic cancer cell lines [59]. A similar study with stemlike cells sorted from 2 breast cancer cell lines found a similar effect of carbon ions combined with cisplatin, with the additional observation that genes related to apoptosis, angiogenesis, and metastasis were also most efficiently inhibited by this combination, when compared with the effects of carbon ions alone or x-rays plus cisplatin [60]. Both these studies concluded that induction of irreparable complex DNA damage induced by carbon ions plus chemotherapy agents triggered increased stem cell death due to apoptosis, but further studies are needed to optimize the combination of ion therapy with chemotherapy for specific tumors.

**Getting More from Expression Studies**

Although many studies of particle irradiation have used curated gene sets aimed at addressing specific questions, others have used whole genome techniques. When well documented, whole genome studies have great potential to contribute to research areas far beyond the scope of the initial investigation. To aid the interpretation and further use of whole genome data, the Minimum Information About a Microarray Experiment (MIAME) standard was developed [61]. Many funding agencies and journals encourage or require MIAME compliance, which may include deposition of original data in a publically accessible database such as the Gene Expression Omnibus (GEO) [62] or ArrayExpress [63]. The particle radiation therapy field currently appears to be underserved in this area of data sharing.

This can be illustrated by looking at the small number of published whole genome studies of carbon ion response as an example. These studies cover a wide range of ion energies, average LET, doses, times post exposure, cell or tumor types, and experimental design (Table 1). Given this diversity it is not possible to begin forming a comprehensive picture of responses from these publications. Moreover, most of these studies have reported only results tailored to a very specific question, so that all the additional information potentially gained from the experiment has been lost to the research community. While 4 of the studies in Table 1 have made complete data sets available via the publically accessible GEO database, it is quite surprising that none of the other studies have even provided a complete list of differentially expressed genes from their analyses. This makes it impossible for readers to compare other responses, even of single genes, between studies, or to begin
looking for consensus responses using parameters that may be present in multiple experiments, but which were not the focus of the authors’ analysis.

For example, Moritake et al [32] were interested in identifying genes in the lung that might be associated with different physiological responses to carbon ion exposure seen in 3 mouse strains. They reported 14 genes that met their relatively stringent selection criteria and differed between the strains, focusing on 2 genes that showed a persistent elevated response in C57BL/6J, the only strain to show late fibrosis. However, since they made their data available in GEO, it could be reanalyzed to ask the same question, using different analysis parameters, or to glean insight into different questions. In a simple analysis of the data using BRB array tools [64], over a thousand genes were found to be significantly responsive ($P < 0.001$; false discovery rate $< 5\%$) in at least 1 of the irradiation conditions tested (Figure 1). This richness of response was not evident from the tight focus of the article. The various expression patterns include genes with strong consistent overexpression in all 3 strains in response to both carbon ions and gamma rays, some genes showing potential batch or experimental effects, and other genes that exhibit strain differences much greater than their radiation response (Figure 1).

Gene ontology enrichment analysis using DAVID [65] showed significant overrepresentation (Bonferroni-corrected $P < 0.05$) of genes in the p53 signaling and intrinsic apoptosis pathways among the genes that were consistently overexpressed across all strains and both radiation qualities. Increased expression of p53 regulated genes is one of the most consistently observed gene expression responses to radiation in normal tissue and tumor cell lines with intact p53 signaling [66, 67], and such responses were also seen in other carbon ion studies in Table 1 [68, 69].

A small set of genes that seemed to vary in expression on the basis of the type of experiment (gamma ray or carbon ion), but not as a function of exposure or strain, was also evident from the heat map. This may be due to a batch effect if the microarray hybridizations were carried out at different times, or to a specific stress to the animals if there were differences in handling or restraint during the different types of irradiation. Since the effect extended to only a small number of genes and did not appear to affect the radiation-responsive genes that were the focus of the study, this is not a major concern. However, this does underline the importance of conducting matched controls appropriate for each irradiation condition to mitigate against confounding by any such unforeseen responses.

Potential strain differences could be further explored by using the data to specifically search for genes differentially expressed among unirradiated controls as a function of strain. This analysis yielded 856 genes differentially expressed in the fibrosis-prone C57BL/6 strain compared with the other 2 strains, and 607 genes differentially expressed in the early pneumonitis-prone C3H/He strain compared with the other 2 strains. Gene ontology analysis showed that genes expressed at a significantly higher level in C57BL/6 and at a lower level in C3H/He were significantly overrepresented by genes involved in cell adhesion and extracellular matrix functions, while several immune functions were overrepresented among genes expressed at a lower level in C57BL/6. As such processes could contribute to the strain-specific radiation pathologies observed, baseline gene expression differences may also
be of interest to pursue, in addition to the radiation-responsive genes that were the focus of the original article.

In another example of the usefulness of publically available gene expression data, Yeles et al [70] analyzed data from several studies and were able to overcome differences in experimental design and other confounding factors to develop consensus pathways responding in unexposed bystander cells cocultured with cells exposed to alpha, gamma, and carbon ion irradiation. These examples illustrate some of the potential for data from whole genome expression assays to contribute to addressing questions beyond the originating study.

Conclusion

Overall, most studies have reported a greater impact of particle irradiation on gene expression response when compared to the response to photons. In tumors or cancer cell lines, a part of this enhanced response generally appears to include greater downregulation of specific categories of genes positively associated with tumor progression or metastasis, suggesting a possible added advantage of particle therapy. This response may not be universal, however, and in some cases particle exposure may enhance tumor progression more efficiently than photons. Systematic gene expression studies, in particular those using whole genome approaches, can make a major contribution towards the mechanistic understanding that will guide development of optimized particle therapy. Data sharing should be strongly encouraged by the field in order to expedite this process.

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Figure 1.
Heatmap of genes responding significantly to gamma or carbon ion exposure in the lungs of irradiated mice. Data from Moritake et al [32] were downloaded from GEO (Accession GSE20959) and analyzed with BRB-ArrayTools [64] as previously described [74]. Genes with expression significantly different ($P < .005$; FDR $< 5\%$) from controls after either gamma-ray or carbon ion exposure in each strain were combined and clustered by using BRB-ArrayTools to produce the heatmap, where each row represents an individual gene measured across all animals (columns). Darker shades of blue indicate higher levels of expression. Several expression patterns are evident, such as strain differences that are greater than the radiation response (A), genes with strong induction following both gamma and carbon ion exposure with little strain differences (B), and radiation-responsive genes with apparent batch or handling effects (elevated expression in all gamma experiments including controls) that is similar across strains (C). Abbreviations: FDR, False Discovery Rate; GEO, Gene Expression Omnibus.
Table 1.

Studies of global gene expression response to carbon ions with comparison to photon response.

| Energy (MeV/n) | LET (keV/μm) | Dose (Gy) | Time (h) | Cells or tissues | Study area | Platform | Criteria for DEx<sup>a</sup> | No. genes reported<sup>b</sup> | Data availability | Reference |
|----------------|--------------|-----------|----------|-----------------|------------|----------|-----------------|----------------|----------------|-----------|
| 290            | 50           | 30        | 24       | Mouse tumor     | Genes associated with radiation-induced radioresistance | Codelink whole mouse genome array | P < 05 and 1.5-fold change | NR             | GEO<sup>c</sup> GSE5331 | 71        |
| 290            | NR           | .25, 4    | 12       | A549            | Radiation-induced genes correlated with cancer cell aggressiveness | Ace Gene Human Oligo Chip 30K | 2-fold change | 23 up 22 down | None<sup>d</sup> | 49        |
| 9.8            | 170          | 2         | 4        | A549            | Genes responding to C ions but not x-rays | 12K human house print | 2-fold change (C only, not x-ray) | 49             | None          | 72        |
| 75             | 33.7         | 2         | 8        | PC3             | Radiation-induced genes correlating with recurrence-free survival in patients | Affymetrix Human Gene 1.0 ST | 2-fold change and FDR < 5% | 1145 down 518 up | None          | 50        |
| 290            | 50           | 2         | 1, 3     | Melanoma cell lines (HMV-1, 92-1, Colo679, HMY-II, C32TG, MeWo) | C-ion-induced genes (1) common to 6 cell lines (2) common to 3 of 4 x-ray-resistant lines | Codelink whole human genome array | ANOVA P < .001 | (1) 19 down, 3 up (2) 119 down, 54 up | GEO GSE6630 | 69        |
| 290            | NR           | 10        | 6        | Mouse lung      | Radiation-induced genes implicated in interstrain variation in early pneumonitis after C ions | Agilent Mouse whole genome | P < .001; 3-fold change, FDR < 5% | 14             | GEO (GSE20959) | 32        |
| 290            | 70           | 2         | 2, 4, 8  | Human embryonic lung fibroblast | Radiation-induced genes implicated in microcephaly formation | HiCep | NR | 1 | None | 73        |
| 18.3           | 103          | 10, 50, 250 particles | 2, 6 | Primary human diploid fibroblasts | Signalling between irradiated and bystander cells | Agilent whole human genome | P < .001, FDR < 5%, 1.5-fold change | 874 at 2 h 650 at 6 h | GEO (GSE8993) | 68        |

Abbreviations: LET, linear energy transfer; DEx, differential gene expression; NR, not reported; GEO, Gene Expression Omnibus; GSExxxx, GEO accession number for study; C, carbon; FDR, False Discovery Rate; ANOVA, analysis of variance.

<sup>a</sup>Summary of criteria used in study to determine DEx.

<sup>b</sup>Number of genes reported as differentially expressed after carbon ion exposure, using the stated criteria.

<sup>c</sup>Primary data available. GEO (https://www.ncbi.nlm.nih.gov/geo/).

<sup>d</sup>Primary data not publicly available and no lists of differentially expressed genes published in the study.