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Original article

Identification of a radiation sensitivity gene expression profile in primary fibroblasts derived from patients who developed radiotherapy-induced fibrosis

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ABSTRACT

Background and purpose: During radiotherapy, normal tissue is unavoidably exposed to radiation which results in severe normal tissue reactions in a small fraction of patients. Because those who are sensitive cannot be determined prior to radiotherapy, the doses are limited to all patients to avoid an unacceptable number of severe adverse normal tissue responses. This limitation restricts the optimal treatment for individuals who are more tolerant to radiation. Genetic variation is a likely source for the normal tissue radiosensitivity variation observed between individuals. Therefore, understanding the radiation response at the genomic level may provide knowledge to develop individualized treatment and improve radiotherapy outcomes.

Material and methods: Exon arrays were utilized to compare the basal expression profile between cell lines derived from six cancer patients with and without severe fibrosis. These data were supported by qRT-PCR and RNA-Seq techniques.

Results: A set of genes (*FBN2*, *FST*, *GPRC5B*, *NOTCH3*, *PLCB1*, *DPT*, *DDIT4L* and *SGCG*) were identified as potential predictors for radiation-induced fibrosis. Many of these genes are associated with TGFβ or retinoic acid both having known links to fibrosis.

Conclusion: A combinatorial gene expression approach provides a promising strategy to predict fibrosis in cancer patients prior to radiotherapy.

A primary treatment strategy for cancer control is the use of ionizing radiation (IR) to control and kill tumors. A range of individual enhanced radiosensitivity exists within the human population [1] most without any evident phenotypes, with the exception of a few rare autosomal radiosensitivity syndromes such as ataxia telangiectasia [2], ligase IV [3] Nijmegen breakage syndrome [4], and AT-Like disorder [5]. The normal tissue damage is a major concern for radiation oncologists. Radiotherapy regimens have been developed with the aim of minimizing adverse effects while maximizing tumor control. Given the diversity of individual radiosensitivity among the human population, a primary focus of Radiation Oncology research is to improve treatment outcomes through individualization of radiation treatment. In effect, the few radiosensitive patients limit the maximum dose that can be delivered to all patients. If these patients could be identified prior to treatment, then the majority of patients who are not radiosensitive could be treated with higher radiation doses with the expectation of greater tumor control. Therefore, the ability to predict patient radiosensitivity prior to treatment would have a significant impact on clinical practice.

There are a number of lines of evidence that suggest genetic factors play a major role in the heterogeneity of radiation sensitivity in the population [6–9]. Examples of radiosensitivity in individuals due to defective genes include defects in *ATM*, *NBS1*, *MRE11* and *LIG4* genes [5,10,11], and cells derived from these individuals show radiosensitivity. Also, genes involved in pathways predicted to cause radiosensitivity when dysfunctional (for example, DNA repair genes), show enhanced radiosensitivity in cellular and chromosomal based assays [11–16]. However, the use of cellular and chromosomal *in vitro* assays has not always yielded clear correlations between cellular and clinical radiosensitivity [17–20], and no assay to date can predict clinical radiosensitivity with a high degree of certainty. Advances in genomic technologies have

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enabled large scale gene expression studies to be conducted and offer a promising approach toward the development of a radiosensitivity prediction assay. Some gene expression studies have identified a radiosensitivity signature in different experimental settings [21–26]. These studies have been quite variable in terms of the cell types tested, numbers of patients evaluated, type of genomic platform used, definition of radiosensitivity, tested with or without IR treatment, severity of adverse responses and primary tumor type.

Genomic approaches including gene expression arrays and transcriptome sequencing are promising strategies to identify a characteristic radiosensitivity profile. In the present study, gene expression at the exon level for primary fibroblasts derived from cancer patients who displayed abnormally severe fibrosis following standard radiotherapy has been determined both prior to and after IR. Fibrosis is a normal response to wound healing which involves a large number of proteins that are involved in a fine balance between tissue repair and resolving scar tissue. An imbalance in the basal gene expression levels of genes involved in fibrosis could result in a fibrotic pathology after radiation injury.

Methods

Cell lines and culture

Primary fibroblasts were derived from unirradiated skin biopsies as previously described ([27,28]. Fibroblasts were grown in RPMI media supplemented with 10% FBS and 20 μg/ml gentamicin and incubated in a 5% CO_2 humidified incubator. Fibroblasts were also irradiated with 10 Gy and RNA was isolated at 4 h post-IR. A Cs^{137} source with a dose rate of 1.7 min/Gy was utilized to irradiate the cells at room temperature [28]. Radiosensitive patients were defined as those who had experienced an abnormally severe adverse reaction to radiotherapy (Radiation Therapy Oncology Group (RTOG) grade $\geqslant 3$). Samples from 8 patients who experienced late adverse reactions and 6 control patients (individuals who did not experience any adverse RT reactions) were analyzed (Table 1) [29,30]. All patients have given written informed consent and studies have been approved by the Peter MacCallum Cancer Centre and Monash University Ethics Committees.

RNA isolation

Cells were grown to a density of 1×10^7, pelleted, resuspended in 3 ml PBS and an equal volume of Trizol (Invitrogen, Carlsbad, CA, USA) was added, mixed and the aqueous layer was mixed with an equal volume of 70% ethanol and added onto a RNeasy column (Qiagen, Venlo, The Netherlands). The RNA extraction was continued by using the RNAeasy method as per the manufacturer's recommendation except starting with the addition of the sample of Buffer RW1. RNA concentration and integrity was determined by analyzing on a bioanalyzer 6000 Nano Labchip as per the manufacturer's recommendations (Agilent, Santa Clara, CA, USA). RNA was determined to be of high enough quality if a minimum RIN of 8.5 was obtained.

Exon arrays

GeneChip Human Exon 1.0 ST Array analysis was performed as per the 'GeneChip Whole Transcript (WT) Sense Target labelling assay Manual' (Affymetrix, Santa Clara, CA, USA). The rRNA from 1 μg of total RNA was reduced using a RiboMinus Human/Mouse Transcriptome Isolation Kit (Invitrogen, Carlsbad, CA, USA) [28,31]. The array data utilized in this paper have been uploaded to the gene expression omnibus database: accession number GSE26841. The following files relate to this manuscript: GSM660490, GSM660491, GSM660494 GSM660498, GSM660506, GSM660507, GSM660509, GSM660510, GSM660512, GSM660516, GSM660523, GSM660524, GSM660526, GSM660527, GSM660529, GSM660530, GSM660533, GSM660537, GSM660545, GSM660549, GSM660556, GSM660557, GSM660559, GSM660560. The analysis utilized the 'core set' which consisted of 228,000 probe set regions covering 17,882 genes, with approximately 40 probes per gene.

Exon array analysis

For this investigation we have analyzed the 'core set' that is defined by over 200,000 probe set regions (Affymetrix.com). Assessment of array quality was determined using Expression Console (Affymetrix.com). Gene expression was assessed using R, normalized using RMA and analyzed using Significance Analysis of Microarrays (SAM; [32]. Differential expression was also deter-

Table 1
Clinical information about the cancer patients involved this study.

Cell line	Cancer site	Histology	Adverse reaction	RTOG	Total dose received[a]	Boost[b]	Chemo	Smoker[e]	Follow-up[f]
C1	Breast	IDC	None	0–1	60 Gy	Yes	None	No	4.5
C2	Breast	IDC	None	0–1	60 Gy	Yes	None	No	1
C3	Breast	Medullary	None	0–1	60 Gy	Yes	None	No	1.5
C4	Breast	IDC	None	0–1	60 Gy	Yes	None	No	4
C5	Breast	IDC	None	0–1	61 Gy	Yes	None	No	6
C6	Breast	IDC	None	0–1	61 Gy	Yes	None	No	6.5
R1	Breast	IDC	Fibrosis	3	61 Gy	Yes	None	Yes	2.5
R2	Breast	IDC	Fibrosis	3	18 Gy[c]	No	None	No	N/A[g]
R3	Breast	Medullary	Fibrosis	3	61 Gy	Yes	None	No	11
R4	Prostate	AC	Proctitis	3	66 Gy	Yes	None	No	3
R5	Breast	IDC	Fibrosis	3	61 Gy	Yes	None	No	6.5
R6	Breast	IDC	Fibrosis	3	60 Gy	Yes	None	No	1
R7[h]	Breast	IDC	Fibrosis	3	60 Gy	Yes	Yes[d]	No	1.5
R8[h]	Breast	N/A	Fibrosis	3	N/A	N/A	N/A	N/A	N/A

IDC: infiltrating ductal carcinoma; AC: adenocarcinoma; RTOG: Radiation Therapists Oncology Group; N/A: not available.
[a] Initial regime plus boost. All fractionated radiation therapy (46 or 50 Gy) was planned for 5 weeks.
[b] Boost was 10 or 15 Gy over 5 weeks.
[c] Cessation of radiotherapy for patient RS2 occurred due to an adverse acute response; the patient later developed fibrosis.
[d] Adjuvant cyclophosphamide, methotrexate and 5-flurouracil.
[e] Patient samples C4, C5, R4 and R5 were Ex-smokers for >10 years before diagnosis. R1 ceased smoking from diagnosis according to patient records.
[f] The last follow-up recorded in years after radiotherapy.
[g] The exact time is unavailable but was greater than 6 months.
[h] Fibroblasts derived from these patients were not used in exon array experiments.

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