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Radiation-induced effects on gene expression: An in vivo study on breast cancer

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Abstract

***Background and Purpose*: Breast cancer is diagnosed worldwide in approximately one million women annually and radiation therapy is an integral part of treatment. The purpose of this study was to investigate the molecular basis underlying response to radiotherapy in breast cancer tissue.**

***Material and Methods*: Tumour biopsies were sampled before radiation and after 10 treatments (of 2 Gray (Gy) each) from 19 patients with breast cancer receiving radiation therapy. Gene expression microarray analyses were performed to identify in vivo radiation-responsive genes in tumours from patients diagnosed with breast cancer. The mutation status of the *TP53* gene was determined by using direct sequencing.**

***Results and conclusion*: Several genes involved in cell cycle regulation and DNA repair were found to be significantly induced by radiation treatment. Mutations were found in the *TP53* gene in 39% of the tumours and the gene expression profiles observed seemed to be influenced by the *TP53* mutation status.**
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With 1 million new cases diagnosed annually worldwide, breast cancer is by far the most common female cancer, comprising about 20% of all new cancers in women. In Norway around 2500 cases are diagnosed annually, and radiotherapy remains a key component of modern multimodal anticancer treatment approaches. Most clinically relevant improvements in radiation oncology have up to now been based on optimized radiation technology, but biology-based approaches could further boost the efficacy of modern radiation oncology.

From a clinical viewpoint, large variations both in effect of treatment and development of side-effects are seen, but little is known as to why this is the case. This project was initiated to increase our knowledge about the molecular biology underlying differences in tissue response to radiation.

The success of radiotherapy depends on its ability to selectively kill tumour cells. Ionizing radiation (IR) exposure of mammalian cells causes a spectrum of lesions within the cell. At the DNA level, these lesions include DNA single- and double-strand breaks (SSBs, DSBs), DNA base damage and DNA—protein crosslinks, with DSB considered as the most important lesion triggering cell death responses. Aside from DNA damage, IR also causes a spectrum of other lesions in cellular macromolecules due to reactive oxygen species (ROSs).

The eukaryotic strategy to deal with damaged DNA consists of three components: (1) activation of a cellular system recognizing and sensing the damaged DNA, (2) a period of damage assessment, and (3) the implementation of the appropriate response, DNA repair or cell death [20]. The damage sensors stimulate specific signalling processes that activate transcription factors, such as SP1, p53 and NF-kb [6]. These processes are not activated in a simple linear fashion because the damage recognition elicits multiple signal transduction cascades that can trigger both repair and apoptotic processes [8,20].

P53 has been recognized as an important checkpoint protein, acting as the guardian of the genome [9]. This tumour suppressor gene plays a critical role in maintaining genomic integrity after cellular stress by acting as a transcription factor for a number of downstream genes. These genes

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may mediate either apoptosis or cell cycle arrest followed by repair, thereby preventing propagation of damaged DNA [12]. The p53 protein expression is tightly regulated and remains low in unstressed cells [13]. It is a short-lived protein and is stabilized and activated by a wide range of cellular stresses [13]. In response to DNA damage, the serine/threonine protein kinase ATM is activated: ATM then phosphorylates several downstream molecules, including p53 [3,18]. The p53 protein is then activated and transcriptionally controls target genes that influence multiple response pathways, triggering a diversity of responses to IR in mammalian cells.

TP53 mutations are found in 20–40% of breast carcinomas, and this mutation has been shown to influence their prognosis [5,10].

The tools of the post-genomic era enable high-throughput studies of the multiple changes resulting from the interplay of radiation signalling pathways. Transcriptional responses to radiotherapy have not been well characterised in vivo in human tumours. To obtain a genome-wide portrait of the transcriptional response to radiotherapy in human breast tumour cells, we performed expression analyses using Agilent oligonucleotide microarrays. Since *TP53* plays a key role in DNA damage control and is mutated/inactivated in a substantial fraction of breast tumours, we wanted to explore whether combining *TP53* mutational status with large-scale expression analyses could help unravel the additional regulatory factors contributing to the complex patterns involved in radiation response.

Materials and methods

Patients

Patients with ulcerating breast cancer (stage III and IV) or local relapse from breast cancer were invited to participate in this study. Our Institutional Review Board and the Regional Ethical Committee approved the study and informed consent was signed by the patients included. Tumour tissue was biopsied before radiation and after 10 treatments (totally 20 Gy) and stored at −80 °C. The pathologist (JMN) evaluated the tumour content in the 21 biopsies with sufficient material, to assure that these samples contained enough tumour tissue for further analyses. In order to minimise the trauma to the patients, small biopsies were taken.

An overview of the histopathological and clinical diagnosis of the patients is found in Table 1.

Microarray expression analyses

Briefly, total RNA was isolated using TRIzol solution (Invitrogen, Carlsbad, CA) or RNeasy Mini Kit (Qiagen) after homogenization. The concentration of total RNA was determined and the integrity of the RNA was assessed using a 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). The total RNA was then amplified and labelled using an optimized protocol. (http://www.chem.agilent.com). Tumour RNA was labelled with Cy5 (red fluorescence) and RNA from Universal Human Reference (Stratagene, La Jolla, CA) was labelled by Cy3 (green fluorescence). The hybridization of amplified and labelled cRNA to oligo microarrays was performed according to the manufacturer's instructions (http://www.chem.agilent.com). Briefly, labelled cRNAs were hybridized overnight to Agilent Human 1A oligo microarrays containing 22,153 features representing 20,173 genes. After washing, the microarrays were scanned in an Agilent microarray scanner (in the Stanford core facility). Images were processed using Agilent Feature Extraction software, providing normalized Cy-3 and Cy5 channel intensity values for each spot on an array. The default settings were used for all options. Quality control algorithms in the software detected poor quality spots that were excluded from analysis. The primary data tables and the

Table 1
Clinical and pathological characteristics of the patients

ID	Histology	Grade	ER	PR	HER2	Stage	*TP53* mut
101	IDC	III	neg	neg	pos	III	mut
102	IDC	nd	pos	pos	nd	IV	wt
103	IDC	II	pos	pos	nd	IV	wt
104	IDC	III	neg	neg	nd	III	nd
105	IDC	III	neg	neg	nd	III	mut
106	ILC	nd	pos	pos	nd	III	wt
107	IDC	II	pos	pos	nd	IV	mut
108	IDC	III	pos	pos	nd	IV	wt
109	IDC	III	neg	neg	neg	IV	mut
110	IDC	III	neg	neg	pos	III	mut
112	IDC	III	pos	pos	nd	IV	wt
113	Muc Carc	II	pos	neg	nd	IV	wt
114	IDC	nd	pos	pos	nd	IV	wt
117	IDC	III	neg	neg	pos	III	mut
118	ILC	II	neg	neg	nd	III	wt
119	IDC	III	neg	neg	Nd	nd	mut
120	ILC	II	pos	pos	neg	IV	wt
121	IDC	II	pos	pos	neg	IV	wt
122	IDC	II	pos	neg	neg	nd	wt

IDC, infiltrating ductal carcinoma; ILC, infiltrating lobular carcinoma; neg, negative; pos, positive; mut, mutated; wt, wild type; nd, not determined; Muc carc, mucinous carcinoma.

image files are stored in the Stanford Microarray Database (SMD; http://smd.stanford.edu/) [4].

Data processing and analysis

Hierarchical unsupervised clustering [7] was used to cluster genes and tumours based on the similarity in the pattern of gene expression. The resulting dendrogram was visualized using TreeView software (http://rana.lbl.gov/EisenSoftware.html). This clustering was based on 4126 oligos whose expression varied with two standard deviations (SD) from the mean in at least one array and with data present in more than 80% of the samples. Thereafter, clustering was performed using a gene list of IR-induced (632 genes) and repressed (859 genes) genes ([14] suppl. information) identified by Significance Analysis of Microarrays (SAM) (http://www-stat.stanford.edu~tibs/SAM/) [17]. One thousand and seventy-two of these genes were present on the Agilent arrays used in this study.

Real-time PCR

Quantitative real-time PCR was performed using ABI PRISM 7900 HT Sequence Detection System (Applied Biosystem Inc., Foster City, CA) and specific locked nucleic acid (LNA probes from Universal Probe Library (Roche Diagnostics)). Intron-spanning primer pairs were designed using the online Universal Probe Library Array Design Center. Reverse transcription was performed on 1 μg total RNA for 1 h at 42 °C, using an oligo(dT) 24 primer and Superscript II reverse transcriptase (Life Technologies). Cycling was performed under the default conditions of ABI PRISM 7900 HT Sequence Detection System. Each PCR was run in triplicate to obtain mean of relative expression values. All samples were normalized to PMM1, as this gene shows minimal expression variation of signal intensity between the groups.

Statistical analyses

We applied SAM to search for candidate genes that differed significantly in RNA expression between the irradiated samples and the non-irradiated samples. SAM identifies genes with statistically significant changes in expression by performing a set of gene-specific *t*-tests. For each gene a score is calculated based on its change in expression relative to the standard deviation in multiple samples. SAM also provides an estimate of the false discovery rate (FDR) (percentage of genes identified by chance). Primarily we subjected the 4126 genes from the filtering described above to the SAM analysis. Thereafter we did the same analysis on the samples using the gene list described in Rieger et al. [14], leaving 289 oligos whose expression varied with two standard deviations (SD) from the mean in at least one array and with data present in more than 80% of the samples for the SAM analysis.

TP53 mutation analyses

Direct sequencing of cDNA was applied in order to analyse the non-irradiated biopsies for *TP53* mutations. Briefly, the cDNA was prepared with use of total RNA and reverse transcribed by the GeneAmpRNA PCR Core Kit (Applied Biosystems), amplified by PCR using primers as described in Table 2 and was thereafter submitted to direct sequencing with standard dideoxy sequencing reaction using the BigDye® Terminator v1.1 Cycle Sequencing kit with AmpliTaq FS and an ABI 3730 sequencer (Applied Biosystems (AB)). All sequence analyses were repeated with a new PCR for confirmation. Primer sequences are shown in Table 2. Analyses were done using SeqScape Software v5.2 as described by Applied Biosystems (AB). All sequences were evaluated by two different persons to assure quality control.

Results

Samples were collected from 19 patients, and total RNA was successfully isolated from 19 of the tumours collected before radiotherapy and from 17 of the samples collected post-treatment. We did not get enough RNA for further analyses from two post-treatment samples (not evaluated by pathologist due to small biopsies). Clinical characteristics are shown in Table 1.

Unsupervised hierarchical clustering showed that the before- and after-samples from the same patient tended to cluster together (Fig. 1). To avoid the possible bias of letting each patient count twice in the clustering, the samples with both before and after sample were weighted with a weight of 0.5 in the clustering analyses, and the clustering result was unaffected by this.

To identify genes induced or repressed by IR in vivo, we applied SAM [17]. For each gene, the expression levels of the un-irradiated samples were compared with the irradiated samples. Seven genes (*DDB2*, *CDKN1A*, *GDF15*, *GPX1*, *CD14*, *FLJ32009*, and *CD163*) were found induced, and none repressed, with an FDR of 34.5%.

Our next analysis was supervised by a gene list generated by Rieger and co-workers [14]. They used expression microarrays to measure transcriptional response to radiation in lymphoblastoid cells derived from 14 patients experiencing acute radiation toxicity and 43 controls. They reported 1491 IR-responsive genes, and 1072 of these were measured on

Table 2
Primer sequences of the TP53 gene exon 3–11

P53	Forward primer (5′)	Reverse primer (3′)
PCR ampl	GTG ACA CGC TTC CCT GGA TTG	AGT GGG GAA CAA GAA GTG GAG
Sequence 1	GAG CCG CAG TCA GAT CCT AG	
Sequence 2	ACC TAC CAG GGC AGC TAC GG	
Sequence 3	CCT CCT CAG CAT CTT ATC CG	

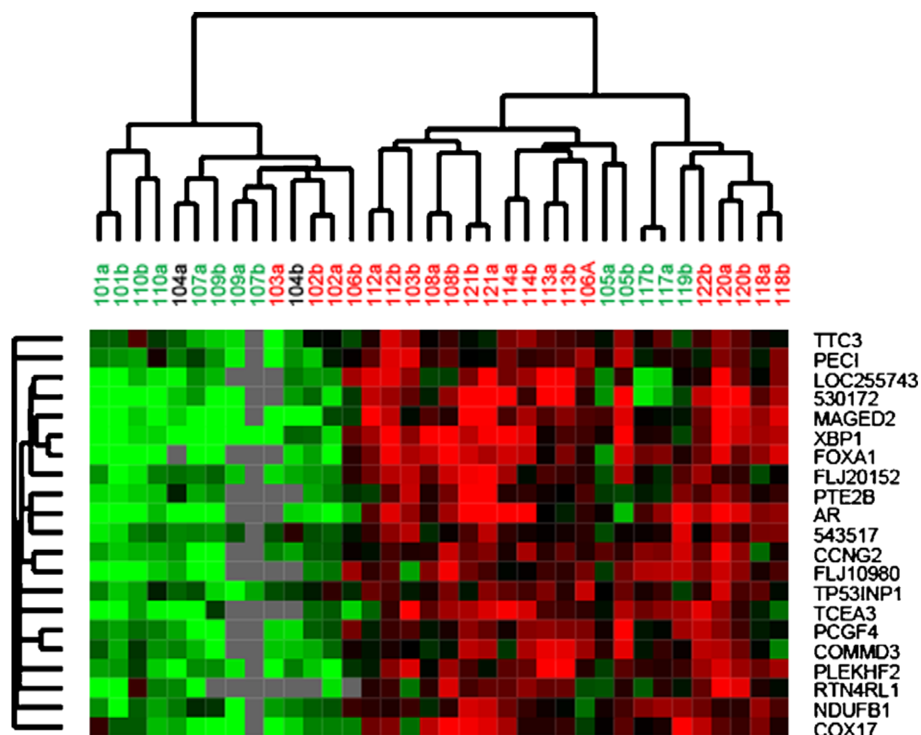

Fig. 1. Unsupervised hierarchical clustering using 4126 genes after filtering as described. TP53 mutated samples are coloured green and wild-type TP53 are in red.

the Agilent arrays we have used. Using these 1072 genes and filtering as described above, 289 clones were the basis of the following SAM analysis. The gene expression in the non-irradiated samples and the irradiated samples was compared using SAM, and five genes were found to be significantly induced in vivo by the radiotherapy. The genes are *DDB2*, *CDKN1A*, *GDF15*, *GPX1* and *PLK3*, with an FDR of 0%. The top four genes were identical both when using all the genes in our analysis, and when using genes previously reported to be radiation influenced [14].

The genes *DDB2* and *CDKN1A* were analysed by RT PCR, to validate that these genes generally are induced by irradiation. In addition the findings seem to indicate that the samples with intact p53 function have a greater induction of both genes than samples with a *TP53* mutation. Sample 101, with a missense mutation in codon 248, did not reveal an induction after irradiation. Details are shown in Table 3.

The *TP53* mutation analyses revealed mutation in seven of the 18 samples (39%) successfully analysed. The mutations detected were mostly missense mutations, and the data are shown in Table 3. Sample number 121 revealed a silent mutation (codon 222) and is in Fig. 1 counted as wild type. When taking the *TP53* mutational status into account, the unsupervised hierarchical clustering of all the samples indicates that the *TP53* mutation status has an impact on the overall expression profile of the tumours.

Discussion

Breast cancer affects many women worldwide, and radiotherapy is an important part of the treatment. However, clinicians see a wide range of effects and side-effects, but the biology underlying tissue responses is not yet fully understood. We have performed expression microarray analyses and detected genes induced by the radiotherapy. *DDB2* and *CDKN1A* were the genes most significantly induced. *DDB2* is involved in DNA repair, is regulated by *TP53*, and transcriptionally activated by the tumour suppressor BRCA1 [16]. *DDB2*-deficient mice have shown a tumour-prone phenotype [19]. *CDKN1A* is a cell cycle regulator, and has been shown induced in patients receiving whole body irradiation [2]. Our findings indicate that the levels of these two genes were induced by irradiation, and most profoundly so in the samples with wild-type *TP53*.

We found the same genes induced when using all genes in our analysis, as when using only radiation-involved genes based on the literature. This supports the conclusion that it is a true radiation induction rather than being caused by the passage of time between the two tissue samplings. In addition, both *DDB2* and *CDKN1A* were previously identified as radiation-induced in peripheral white blood cells [2]. Microarray analyses of radiated fibroblasts also find these genes induced by radiation [15]. *GDF15* is a member of the transforming growth factor-β superfamily and regulates tissue differentiation and maintenance. It has recently been shown to be induced by radiation in human colon cancer cell lines [11]. Glutathione peroxidase (*GPX1*) is one of the most important antioxidant enzymes in humans. It has been reported that the protein encoded by this gene protects from CD95-induced apoptosis in cultured breast cancer cells (e.g., see http://source.stanford.edu/). *PLK3* is a cytokine-inducible kinase (CNK) and is a putative serine/threonine kinase. CNK contains both a catalytic domain and a putative

Table 3
TP53 mutations and fold change induction (by RT PCR)

ID	Mutation nucleotide change	Aminoacid change WT>Mut	Effect	DDB2 fold change (after/before)	CDKN1A fold change (after/before)
101	c742 C > T (homo)	Arg > Trp	Missense	0.63	0.51
102	WT			13.68	10.47
103	WT			5.17	0.67
104	ND			ND	ND
105	c841 G > A (homo)	Asp > Asn	Missense	1.67	0.36
106	WT			23.01	207.78
107	c524 G > A (het)	Arg > His	Missense	4.42	218.31
108	WT			0.42	0.96
109	c400 del T (het)	stop in C.169	Deletion	ND	0.18
110	c584 T > C (homo)	Ile > Thr	Missense	0.036	0.003
112	WT			0.719	3.67
113	WT			2.13	9.23
114	WT			1.04	29.48
117	c328del C (het)	>stop in C.122	Deletion	2.52	0.89
118	WT			8.26	23
119	c818 G > A (het)	Arg > His	Missense	ND	ND
120	WT			0.63	5.2
121	c666 G > T (het)	Pro > Pro	Silent	2.34	42.32
122	WT			ND	ND

WT, wild type; Mut, mutated; ND, not determined; homo, homozygote; het, heterozygote; c, coding sequence.

regulatory domain. It may play a role in regulation of cell cycle progression and tumorigenesis (see http://source.stanford.edu/). *PLK3* did not appear in the SAM based on all genes, and this might of course be due to the limited sample number. On the other hand, CD14, CD163 and FLJ32009 did not come up as significantly induced/repressed in the analysis based on the known radiation-induced/repressed genes, even though CD14 and CD 163 are on the arrays used in that study (U95A_v2). FLJ32009 is a sequence described as von Willebrand factor C and EGF domains (www.ncbi.nlm.nih.gov).

DNA repair is widely regulated by *TP53*, and we successfully analysed 18 pre-irradiated samples for *TP53* mutations, finding a high percentage of mutations. We find mainly missense mutations, and this could be because our analyses are based on the cDNA and not the gDNA. However, the frequency of mutations is high, and this might be explained as these are advanced tumours. A previous report [1] indicated a radiation-induced increase in RNA levels of both *DDB2* and *CDKN1A* in epithelial and lymphoid cell lines with intact wild-type *TP53*. However, in cell lines with a *TP53* mutation, this induction was not found. In three of the samples in our series, we find homozygote mutations (101, 105, and 110), with no indications of present wild-type *TP53*. This is rarely found in DNA-analyses, and might be because the mutated cells have a higher expression of the RNA than the wild type.

As shown in Table 3, many of the *TP53* mutated samples revealed no induction of DDB2 and CDKN1A when analysed by RT PCR. Both of these genes are regulated by TP53 in response to ionizing radiation, and it is not surprising that this tissue reaction is altered in p53 incompetent cells. On the other hand, sample number 107 had a missense mutation in codon 175, but also a high induction of both DDB2 and CDKN1A. This could be due to the other not-mutated allele in this tumour, or other genes in the pathway overriding the effect of the mutation. On the other hand, 108, with a wild-type *TP53*, showed no induction of the genes. This could of course be due to inactivation of other genes in the p53 pathway, or the presence of a mutation not detected by our analysis.

In an attempt to unravel the underlying biology behind tissue effects of radiation, in vivo radiation-induced expression changes in tumour biopsies were analysed by microarray analyses. Genes involved in cell cycle regulation and DNA repair were found induced by radiotherapy. Both the similarities and the differences in the expression levels between samples could point to mechanisms behind radiation-induced responses. Larger studies are needed to unravel more of the biology behind radiotherapy effects. To investigate whether these radiation-induced gene expression changes can predict outcome of the radiotherapy, more samples would be needed.

Insights into our understanding of how *TP53* modulates radiosensitivity/resistance in tissues following radiotherapy could elucidate options of exploiting the *TP53* pathway as target in therapy.

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