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Changes in DNA Damage Repair Gene Expression and Cell Cycle Gene Expression Do Not Explain Radioresistance in Tamoxifen-Resistant Breast Cancer

Annemarie E. M. Post,*† Johan Bussink,* Fred C. G. J. Sweep,† and Paul N. Span*

*Radboud University Medical Center, Department of Radiation Oncology, Radiotherapy and OncoImmunology Laboratory, Nijmegen, The Netherlands
†Radboud University Medical Center, Department of Laboratory Medicine, Nijmegen, The Netherlands

Tamoxifen-induced radioresistance, reported in vitro, might pose a problem for patients who receive neoadjuvant tamoxifen treatment and subsequently receive radiotherapy after surgery. Previous studies suggested that DNA damage repair or cell cycle genes are involved, and could therefore be targeted to preclude the occurrence of cross-resistance. We aimed to characterize the observed cross-resistance by investigating gene expression of DNA damage repair genes and cell cycle genes in estrogen receptor-positive MCF-7 breast cancer cells that were cultured to tamoxifen resistance. RNA sequencing was performed, and expression of genes characteristic for several DNA damage repair pathways was investigated, as well as expression of genes involved in different phases of the cell cycle. The association of differentially expressed genes with outcome after radiotherapy was assessed in silico in a large breast cancer cohort. None of the DNA damage repair pathways showed differential gene expression in tamoxifen-resistant cells compared to wild-type cells. Two DNA damage repair genes were more than two times upregulated (*NEIL1* and *EME2*), and three DNA damage repair genes were more than two times downregulated (*PCNA*, *BRIP1*, and *BARD1*). However, these were not associated with outcome after radiotherapy in the TCGA breast cancer cohort. Genes involved in G_1, G_1/S, G_2, and G_2/M phases were lower expressed in tamoxifen-resistant cells compared to wild-type cells. Individual genes that were more than two times upregulated (*MAPK13*) or downregulated (*E2F2*, *CKS2*, *GINS2*, *PCNA*, *MCM5*, and *EIF5A2*) were not associated with response to radiotherapy in the patient cohort investigated. We assessed the expression of DNA damage repair genes and cell cycle genes in tamoxifen-resistant breast cancer cells. Though several genes in both pathways were differentially expressed, these could not explain the cross-resistance for irradiation in these cells, since no association to response to radiotherapy in the TCGA breast cancer cohort was found.

Key words: Treatment resistant; Tamoxifen treatment; Radiotherapy; DNA damage repair; Cell cycle control

INTRODUCTION

Radiotherapy and hormonal treatment (tamoxifen) are both corner stones of breast cancer treatment, and are successful in a large number of patients. However, when resistance to these treatment modalities occurs, adverse outcomes are likely for the patient. Previously we, and others, showed that breast cancer cells cultured to tamoxifen resistance also acquire radioresistance in vitro[1–3]. In the classical breast cancer treatment regimen, where tamoxifen is given after radiotherapy following surgery, this poses no problem for patients. However, neoadjuvant endocrine therapy, such as tamoxifen, is increasingly given to breast cancer patients. Endocrine treatment-induced radioresistance could pose a problem for these patients when they receive radiotherapy later in the treatment schedule[4]. Moreover, patients that receive radiotherapy as treatment for metastasized disease could suffer from reduced efficiency due to earlier adjuvant tamoxifen treatment.

In order to prevent cross-resistance for irradiation in tamoxifen-treated tumors, it is imperative to identify the genes and/or pathways that cause radioresistance in tamoxifen-resistant breast cancer. Several studies have addressed the expression of DNA damage repair genes in tamoxifen-resistant cells. Luzhna et al.[2] described that while after irradiation wild-type MCF-7 cells had decreased levels of genes making up the base excision

Address correspondence to Paul N. Span, Department of Radiation Oncology (874), Radboud University Medical Center, P.O. Box 9101, 6500 HB Nijmegen, The Netherlands. Tel: 0031 2435 92985; Fax: 0031 2436 19652; E-mail: paul.span@radboudumc.nl

repair (BER), homologous recombination (HR), and mismatch repair (MMR) pathways, these genes were not differentially expressed in tamoxifen-resistant MCF-7 cells after irradiation. Moreover, the tamoxifen-resistant cells displayed more efficient repair of double-strand breaks and were less susceptible to apoptosis. Others found that tamoxifen-resistant MCF-7 cells exhibited increased levels of PARP1 and LIG3, both part of the alternative nonhomologous end joining (NHEJ) pathway, and are dependent on this pathway for repair of double-strand breaks. Significantly more yH2AX foci and a large number of genomic aberrations were present in those resistant cells[5]. Another study showed that BRCA1 and its associated protein BARD1 are upregulated in tamoxifen-resistant breast cancer cells, which render the cells resistant to DNA-damaging chemotherapy[6]. Thus, DNA damage repair is reportedly altered in tamoxifen-resistant breast cancer cells, possibly explaining their cross-resistance to radiotherapy.

Next to DNA damage repair genes, differential expression of cell cycle genes might also contribute to the altered radiosensitivity observed in tamoxifen-resistant cells. Tamoxifen treatment decreases the percentage of cells in S phase, while inducing a G_1 block[7–10]. The efficiency of radiotherapy depends on the cell cycle phase cells are in, a phenomenon that has been studied for a long time[11]. The most sensitive phases of the cell cycle are the G_2 and M phases, while cells in the G_1 phase are more resistant to irradiation, and cells in the S phase are the most resistant[12]. Tamoxifen-resistant cells have increased levels of genes regulating the G_1/S transition (*CCNE1*, *CDK2*, and *E2F1*), and higher percentages of tamoxifen-resistant cells were present in S phase compared to parental cells[13]. Also, *CCND1* and *MYC* are reportedly upregulated in tamoxifen resistance, which could lead to G_1/S phase blockade[14]. Higher expression of these genes in tamoxifen-resistant cells could explain the cross-resistance to irradiation.

Here we aim to assess differentially regulated genes in estrogen receptor-positive breast cancer cells cultured to tamoxifen resistance, in order to explain the increased radioresistance observed in these cells. To this end, we analyzed the expression of DNA damage repair genes and cell cycle genes in tamoxifen-resistant breast cancer cells compared to wild-type cells, and validated their predictive power in a large breast cancer cohort in silico.

MATERIALS AND METHODS

Cell Culture

The culturing of estrogen receptor-positive MCF-7 cells (LCG Standards, Teddington, UK) was described previously, including the number of passages and authentication of the cell lines[3]. Tamoxifen-resistant cells were acquired by culturing MCF-7 cells with 4-hydroxytamoxifen (#H7904; Sigma-Aldrich, St. Louis, MO, USA), increasing the dose weekly up to 10 μM[15].

RNA Isolation

The Total RNA Purification kit (Norgen Biotek Corp., Thorold, ON, Canada) was used to isolate RNA. On column DNase treatment (RNase-Free DNase Set; #79254; Qiagen, Hilden, Germany) was performed, all according to the manufacturer's instructions.

RNA Sequencing

RNA sequencing was performed on wild-type MCF-7 and tamoxifen-resistant MCF-7 cells as described previously[3].

Patients: TCGA Database

Data from the Cancer Genome Atlas (TCGA) project's breast cancer cohort (https://portal.gdc.cancer.gov/projects/TCGA-BRCA) were analyzed for expression of genes of interest and associated to patient outcome after radiotherapy. Data were accessed and processed with the University of California at Santa Cruz Xena Browser at http://xena.ucsc.edu/. Node-negative and metastasis-free patients were selected and grouped into radiotherapy-treated ($n = 209$) and nonradiotherapy-treated ($n = 194$) patients. Relapse-free survival was assessed.

Statistics

Mean deviation from 1 was calculated for the genes in different DNA damage repair pathways or cell cycle phase with a Student's *t*-test. Xena Browser TCGA data were imported into SPSS (SPSS Inc., Chicago, IL, USA), and Breslow *p* values at median cutoff were calculated for all genes of interest.

RESULTS

RNA sequencing of tamoxifen-resistant cells (MCF-7^{TAM}) and MCF-7 wild-type cells (MCF-7^{WT}) was performed. For further analyses, only protein-coding genes were included that had more than 10 reads in either the wild-type cells or tamoxifen-resistant cells. The relative expression of these genes in tamoxifen-resistant cells compared to wild-type cells was assessed (Fig. 1A). Many genes were not differentially expressed in tamoxifen-resistant cells compared to wild-type cells. However, 487 genes were more than two times increased, and 493 genes were more than two times decreased. The genes with the highest change in expression were 500 times increased, or over 6,000 times decreased in tamoxifen-resistant cells compared to wild-type cells.

Expression of DNA Damage Repair Genes in Tamoxifen-Resistant Breast Cancer Cells

We assessed the expression of genes that are known to be involved in various types of DNA damage repair (https://www.mdanderson.org/documents/Labs/Wood-Laboratory/human-dna-repair-genes.html[16,17]); 148 of these genes that were protein coding and passed our

read–count threshold were further analyzed (Fig. 1B). The genes of interest were subdivided in the following groups: base excision repair (BER), mismatch repair (MMR), nucleotide excision repair (NER), homologous recombination (HR), and nonhomologous end joining (NEHJ) (Fig. 1B). None of the groups were differentially expressed in tamoxifen-resistant cells compared to the wild-type cell lines (two-sided *t*-test compared to mean = 1; BER: $p = 0.5483$, MMR: $p = 0.2744$, NER: $p = 0.4092$, HR: $p = 0.7267$, NHEJ: $p = 0.5107$).

Out of these 148 genes, we identified individual genes with a minimum twofold change in expression (Table 1). Only two DNA damage repair genes (*NEIL1* and *EME2*) were upregulated in tamoxifen-resistant cells, while two other DNA damage repair genes (*BRIP1* and *PCNA*) were downregulated.

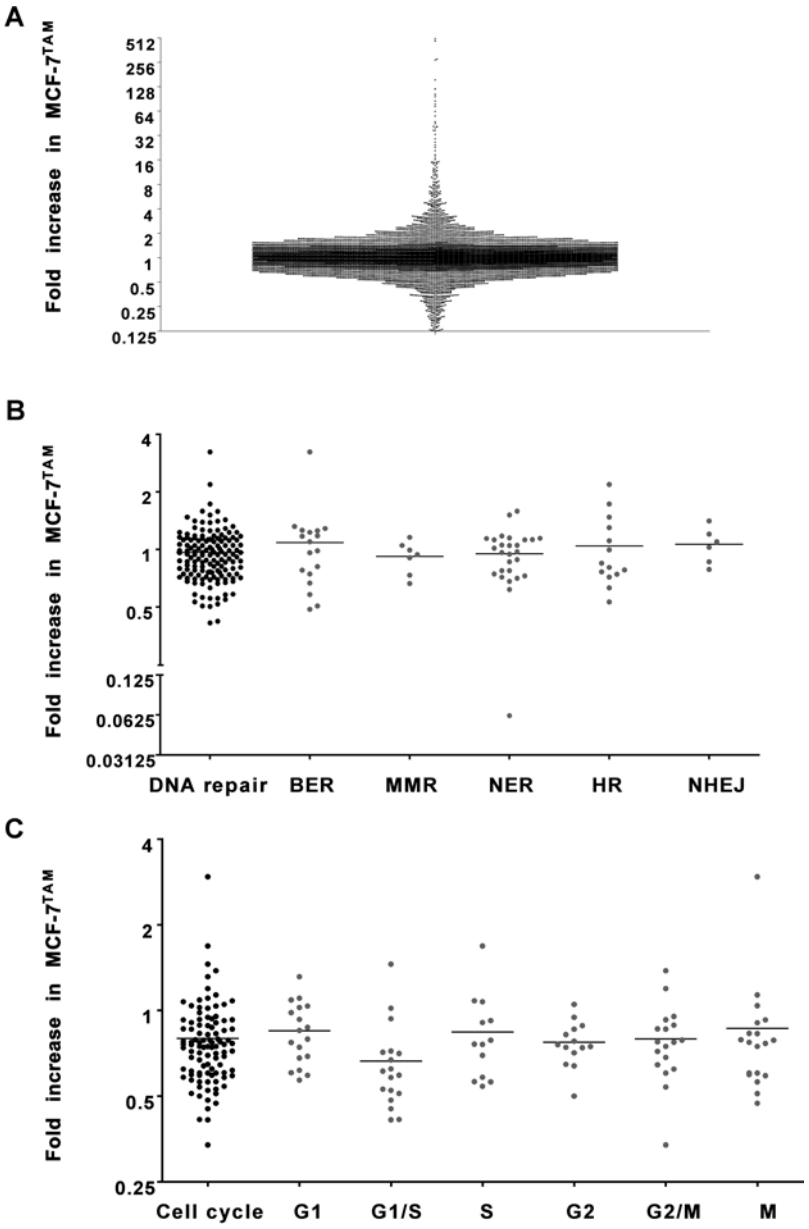

Figure 1. Expression of DNA damage repair genes and cell cycle genes in tamoxifen-resistant breast cancer cells. The expression of (A) all genes, (B) DNA damage repair genes, and (C) cell cycle genes in tamoxifen-resistant MCF-7 cells compared to wild-type cells. For each gene, the fold change in expression levels in MCF-7^{TAM} compared to MCF-7^{WT} is shown (based on normalized read counts), as measured by RNA sequencing.

Table 1. Differential Expression of DNA Damage Repair Genes in Tamoxifen-Resistant Breast Cancer Cells

Gene Name	Gene Function	Fold Change MCF-7^{TAM}
NEIL1	DNA glycosylase, initiation of base excision repair	3.24
EME2	Endonuclease, homologous recombination	2.19
BRIP1	Helicase, BRCA1 interacting	0.42
PCNA	DNA polymerase cofactor, nonhomologous end joining	0.41
LIG3	DNA ligase, base excision repair	1.25
PARP1	Base excision repair	1.18
BRCA1	Homologous recombination	0.63
BARD1	BRCA1 interacting	0.42

List of the differentially expressed DNA damage repair genes in MCF-7^{TAM} and genes that had previously been found to be differentially expressed in tamoxifen-resistant breast cancer cells[5,6]. For each gene, the fold change in expression levels in MCF-7^{TAM} compared to MCF-7^{WT} is shown (based on normalized read counts), as measured by RNA sequencing.

We further included genes that were shown to be differentially expressed in tamoxifen-resistant MCF-7 cells according to the literature: *LIG3*, *PARP1*, *BRCA1*, and *BARD1*[5,6] (Table 1). Of these, only *BARD1* had a more than twofold decreased expression in tamoxifen-resistant MCF-7 cells compared to MCF-7 wild-type cells. In contrast, *BARD1* has previously been found to exhibit increased expression in tamoxifen-resistant MCF-7 cells[6].

Expression of Cell Cycle Genes in Tamoxifen-Resistant Breast Cancer Cells

Besides DNA damage repair genes, differential expression of cell cycle genes in tamoxifen-resistant breast cancer cells might also contribute to radioresistance[12]. One hundred and twenty genes characteristic of six different phases in the cell cycle (20 each for G_1, G_1/S, S, G_2, G_2/M, and M)[18] were analyzed for their expression in the tamoxifen-resistant cells. After selecting for genes that were protein coding and that had more than 10 reads in either the wild-type cells or tamoxifen-resistant cells, 100 genes were left (G_1: 18, G_1/S: 18, S: 13, G_2: 14, G_2/M: 18, M: 19) (Fig. 1C). Gene expression was plotted as fold increase in tamoxifen-resistant MCF-7 cells compared to wild-type MCF-7 cells. Four of the groups showed differential expression in tamoxifen-resistant cells compared to the wild-type cells (two-sided *t*-test compared to mean = 1; G_1: $p = 0.0072$, G_1/S: $p < 0.0001$, S: $p = 0.0907$, G_2: $p < 0.0001$, G_2/M: $p = 0.0017$, M: $p = 0.2806$).

We also analyzed the individual genes for those that had at least two times increased expression or decreased expression in tamoxifen-resistant cells compared to wild-type cells (Table 2). Only one gene was more than two times upregulated (*MAPK13*). Six genes were downregulated in tamoxifen-resistant cells: *E2F2*, *CKS2*, *GINS2*, *PCNA*, *MCM5*, and *EIF5A2*.

Genes upregulated in tamoxifen-resistant cells, according to literature[13,14], were also investigated here. *CCNE1*, *CDK2*, and *E2F1* were marginally (13–41%) downregulated in tamoxifen-resistant cells, but less than our threshold of twofold change. *MYC* and *CCND1* had a decreased expression more than twofold, contrary to the increase that was reported previously[14].

Association of In Vitro Differentially Expressed Genes in a Breast Cancer Patient Cohort

Thus, only a limited number of DNA damage repair and cell cycle control genes exhibit differential expression in tamoxifen-resistant breast cancer cells. To assess the clinical association of the DNA damage repair genes and cell cycle genes with radioresistance, we analyzed

Table 2. Differential Expression of Cell Cycle Genes in Tamoxifen-Resistant Breast Cancer Cells

Gene Name	Gene Function	Cell Cycle Phase	Fold Change MCF-7^{TAM}
MAPK13	Cellular stress-induced signaling protein	M	2.95
E2F2	Cell cycle control	G_1/S	0.48
CKS2	Maintenance of cell wall integrity	M	0.47
GINS2	Initiation of DNA replication	G_1/S	0.45
PCNA	DNA polymerase cofactor	G_1/S	0.41
MCM5	Initiation of DNA replication	G_1/S	0.41
EIF5A2	Cell cycle control	G_2/M	0.34
CCNE1	CDK2 regulator	G_1	0.87
CDK2	Initiation of DNA synthesis	G_1/S	0.71
E2F1	Cell cycle control	G_1/S	0.59
MYC	Cell cycle progression		0.33
CCND1	CDK4/6 regulator	G_1/S	0.31

List of the differentially expressed cell cycle genes in MCF-7^{TAM}. For each gene, the fold change in expression levels in MCF-7^{TAM} compared to MCF-7^{WT} is shown (based on normalized read counts), as measured by RNA sequencing.

data generated by the TCGA Research Network (https://cancergenome.nih.gov/) for relations between the expression of genes of interest and outcome after radiotherapy. Patients with T1–4, N0, M0 tumors were selected, and the cohort was divided in patients that did or did not receive radiotherapy as part of their primary treatment. Patients were dichotomized by the median value of each of the investigated genes. Of the five differentially expressed DNA damage repair genes, none was significantly associated with outcome in patients who received radiotherapy as part of their primary treatment (Table 3). Only expression of *EME2* was specifically associated with poor outcome in patients who did not receive radiotherapy, but not in patients that had received radiotherapy.

The cell cycle genes that were differentially expressed in tamoxifen-resistant breast cancer cells were also examined for an association between their expression and outcome after radiotherapy in the TCGA cohort. None of the more than two times upregulated or downregulated genes were associated with outcome in patients either treated with or without radiotherapy (Table 3).

Therefore, we conclude that none of the differentially expressed genes (DNA damage repair or cell cycle related) are associated with radioresistance in the breast cancer patient cohort investigated.

Table 3. Association of Differentially Expressed DNA Damage Genes and Cell Cycle Genes With Outcome After Radiotherapy

Gene Name	TCGA: RT^-	TCGA: RT^+
DNA damage repair		
Increased		
EME2	0.03	0.37
NEIL1	0.44	0.10
Decreased		
*PCNA**	0.56	0.38
BRIP1	0.19	0.30
BARD1	0.78	0.32
Cell cycle		
Increased		
MAPK12	0.31	0.16
Decreased		
E2F2	0.74	0.47
CKS2	0.62	0.77
GINS2	0.40	0.26
*PCNA**	0.56	0.38
MCM5	0.67	0.33
EIF5A2	0.37	0.89
CCND1	0.28	0.68
MYC	0.15	0.08

For each of the DNA damage genes and cell cycle genes that were differentially expressed in MCF-7^{TAM}, the *p* values of the association with relapse-free survival in patients treated with or without radiotherapy in the TCGA breast cancer cohort are depicted, based on the Breslow test.
**PCNA* is both a DNA damage repair and a cell cycle gene.

DISCUSSION

Here we aimed to identify possible mechanisms of radioresistance in acquired tamoxifen-resistant breast cancer cells by investigating the expression of DNA damage repair genes and cell cycle genes in breast cancer cells cultured to tamoxifen resistance. Increased expression of genes that stimulate DNA damage repair could mean that these are responsible for radioresistance observed in these cells. Conversely, decreased expression of genes that inhibit DNA damage repair could also lead to radioresistance. Moreover, increased expression of cell cycle genes that correspond to radioresistant parts of the cell cycle could explain radioresistance observed in tamoxifen-resistant cells. Interestingly, cross-resistance for irradiation was previously observed in hormone treatment-insensitive prostate cancer cells. These show increased radioresistance and upregulated genes involved in cell cycle arrest and DNA damage repair, suggesting common mechanisms might be involved in various hormone-sensitive cancers[19].

None of the known pathways involved in DNA damage repair (BER, MMR, NER, HR, or NEHJ) were as a whole differentially expressed in the tamoxifen-resistant cells. Luzhna et al. also investigated the expression of genes in DNA repair pathways in tamoxifen resistance, albeit after irradiation, and found that genes in these pathways are not up- or downregulated as well[2]. Interestingly, proteins involved in DNA damage repair are poor prognostic factors in estrogen receptor-positive breast cancer patients treated with endocrine therapy[17]. Therefore, differential expression of DNA damage repair genes could merely be associated with the occurrence of tamoxifen resistance, and not specifically with the radioresistant phenotype. This paper also showed that estrogen receptor-positive breast cancer patients have increased levels of damaging mutations in NER, BER, and NHEJ genes[17].

The specific DNA damage repair genes that were upregulated or downregulated in tamoxifen-resistant cells in our current study cannot explain radioresistance observed in those cells. Although NEIL1 and EME2 (both >2-fold increased RNA expression in tamoxifen resistance) are both stimulators of DNA damage repair[20,21], we did not find an association of their gene expression with outcome after radiotherapy in the TCGA patient cohort, meaning that the gene is not likely to induce radioresistance in patients. *BRIP1* and *PCNA* were downregulated in tamoxifen-resistant breast cancer cells. Since these are both stimulators of DNA damage repair as well[22,23], their decreased expression does not explain radioresistance observed in these cells. BRIP1 was associated with the repair of DNA double strand breaks, as evidenced by assessment of H2AX foci,

after chemotherapy[24]. Its lower expression in the tamoxifen-resistant cells in this study is therefore unclear.

Interestingly, PCNA is involved in DNA damage repair as well as in cell cycle regulation. In a study with radiotherapy-treated oral cancer patients, low expression of PCNA was associated with a better patient survival[25], which indeed points toward an opposite role for PCNA in radioresistance than its expression in tamoxifen-resistant cells in this study suggests. However, in pancreatic tumor cells, *PCNA* was increased in cells treated with rapamycin, which was associated with decreased radioresistance[26]; in another study PCNA inhibition increased the number of double-strand breaks after treatment with DNA-damaging chemo agent cisplatin and therefore sensitized cells to this treatment[27]. PCNA may therefore have a dual role, either promoting or inhibiting DNA damage repair, and this may differ in different cancer models.

A BRCA1-interacting protein, *BARD1*, also showed decreased gene expression in tamoxifen-resistant cells, as well as *BRCA1* itself, which had a slightly decreased expression, contrary to previous reports[6]. We did not observe an increased expression of *LIG3* or *PARP1*, as Tobin et al. did[5]. This shows that even though some aspects of tamoxifen resistance are consistent when creating tamoxifen-resistant cells from wild-type MCF-7 cells, heterogeneity remains an important issue.

Cells are relatively resistant to irradiation in G_1 and S phases, since DNA damage repair genes are highly expressed in those phases to guarantee correct DNA replication. We found a decrease in genes related to G_1 and G_1/S phases, which is not in line with the radioresistant phenotype of these cells. The only increased gene was *MAPK13*. Although it is present in the M phase, a relative radiosensitive phase, it has previously been associated with paclitaxel resistance in breast cancer[28], and it is expressed in radioresistant gynecological cancer stem cells[29]. One of the genes with decreased expression (*E2F2*) is a mediator of apoptosis that is induced after DNA damage[30,31]. Its decreased expression could lead to a decrease in radiation-induced apoptosis, and therefore contributes to radioresistance in tamoxifen-resistant cells. CKS2, which slows down the cell cycle in order to allow repair of DNA damage[32], is associated with enhanced sensitivity to different chemotherapeutic agents when overexpressed[33], which could correspond to enhanced radioresistance in tamoxifen-resistant breast cancer cells where it is downregulated. However, in another study high expression levels of CKS2 were associated with decreased overall survival in breast cancer patients, which is contrary to these data[34]. *MCM5* and *GINS2* showed decreased expression in the tamoxifen-resistant cells. These genes are essential for DNA replication[35]. *MCM5* is associated with worse outcome in patients when highly present in breast cancer patients[36]. *GINS2* was previously associated with tamoxifen resistance when higher expressed in breast cancer patients, in contrast to what we found[37]. In another study, *GINS2* knockdown was found to induce apoptosis[38]. These observations are contrary to the implications that our findings here have. *EIF5A2* was earlier found to be upregulated in radioresistant colorectal cancer cell lines[39], contrary to our findings, and high levels of EIF5A2 were associated with poor outcome after chemoradiotherapy in nasopharyngeal carcinoma patients[40]. In breast cancer high expression levels of EIF5A2 were associated with chemoresistance as well[41]. Finally, *CCND1*, a regulator of G_1/S transition[42], and *MYC* were also downregulated in tamoxifen-resistant breast cancer cells. CCND1 overexpression was previously associated with tamoxifen resistance[43,44], while it was also associated with increased radiosensitivity in MCF-7 cells[45]. The latter is in line with our data. However, *CCND1* knockdown in prostate cancer cells sensitized them to irradiation[46]. Downregulation of MYC has previously been shown to impair cell cycle progression[47]. High expression levels of *MYC* were present in radioresistant breast cancer cells[48] and similarly in docetaxel-resistant lung cancer cells that were cross-resistant to irradiation[49].

Many of the genes that were differentially expressed in breast cancer cells are associated with radiosensitivity. However, we did not find an association between the expression of the genes that were differentially expressed in tamoxifen-resistant breast cancer cells and outcome after radiotherapy in a breast cancer patient cohort. In some cases, our findings were contradictory to what others found in terms of association with radiosensitivity. This can partly be explained by the fact that we found these genes to be differentially expressed after chronic tamoxifen treatment. This change in expression could be an effect of this treatment alone and not involved in the cross-resistance for radiotherapy we observed. Therefore, the effect of these genes on radiosensitivity should indeed be further investigated in tamoxifen-resistant cells.

Other pathways involved in radioresistance ought to be investigated for their role in the cross-resistance observed in tamoxifen-resistant cells, such as hypoxia, one of the three crucial factors in radioresistance, next to proliferation and DNA damage repair[50]. Tamoxifen has been shown to induce hypoxia in MCF-7 xenografts[51]. Therefore, the role hypoxia-induced genes play in radioresistance in tamoxifen-resistant breast cancer should be further investigated. Moreover, in vitro studies may lack information that is crucial for therapy outcome in patients. Other cells in the tumor microenvironment can also contribute to responses to treatment. Another related factor is neovascularization, which is known to codetermine the response to cancer treatment[52]. Therefore, we wanted to confirm the results in clinical datasets, to establish correlations of DNA damage repair genes and cell cycle genes on patient outcome. Finally, the results obtained from retrospective clinical studies may be

biased by the fact that patients who have been treated with radiotherapy differ from patients who have not been thus treated. Therefore, more clinical data are necessary before final conclusions about the role of these DNA damage repair genes and cell cycle genes can be drawn.

CONCLUSIONS

We identified differentially expressed DNA damage repair genes and cell cycle genes in tamoxifen-resistant breast cancer cells. Many of these are related to radiosensitivity according to literature. However, a direct relation between these genes and radioresistance could not be identified, since none of them was associated with outcome after radiotherapy in a breast cancer patient cohort. Thus, changes in DNA damage repair or cell cycle genes do not explain cross-resistance of tamoxifen-resistant breast cancer for radiotherapy and are not likely targets to preclude the occurrence of this cross-resistance.

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