Acta Oncologica, 2015; 54: 1665–1702

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LETTERS TO THE EDITOR

Validation of a gene expression profile predictive of the risk of radiation-induced fibrosis in women treated with breast conserving therapy

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To the Editor,

Today, breast conserving surgery (BCS) followed by radiation therapy (RT) is the primary choice of treatment for breast cancer patients in Denmark. One of the disadvantages of breast conserving therapy is the risk of developing late radiation-induced normal tissue injury. Subcutaneous fibrosis, in particular, is known to cause increased density and hardening of the breast tissue, occasionally leading to disfiguration of the breast, impaired body image and chronical pain, leaving the patient with discomfort and anxiety [1,2]. In addition to total radiation dose and fractionation scheme [3], different factors predispose to the development of radiation-induced normal tissue injury, smoking habits and coexisting morbidity (e.g. collagen vascular disease and diabetes mellitus) [4]. In the clinical setting, great variability in the susceptibility to radiation-induced normal tissue injury reveals itself when patients comparable in treatment and with comparable predisposing factors may experience different levels of late normal tissue reactions, such as fibrosis or telangiectasia [3–5]. This has led to studies exploring the dose response relationship for radiation-induced toxicity [6], but has also launched the search for intrinsic cellular and genetic radiation sensitivity in the need for ways to reduce or avoid the long-term side effects after radiotherapy [5,7,8]. Different approaches have been explored to develop predictive tests for identification of patients with increased risk of radiation-induced subcutaneous fibrosis, including single nucleotide polymorphisms (SNPs) [9] and gene expression analysis [10,11]. We have developed and validated a predictive gene expression profile that identifies the risk of radiation-induced fibrosis [12–14].

In a previously published retrospective follow-up study on 214 Danish breast cancer patients [15] treated from 1989 to 2002, we examined the frequency and degree of late morbidity related to BCT and demonstrated moderate to severe fibrosis (grade II–III) in the residual breast in 23% of the patients 7–20 years after treatment. All patients in the study were seen at a single visit, and together with the physical examination, patients consented to having a skin biopsy taken from the upper arm. Using a previously described predictive test for radiation-induced fibrosis, based on the gene expression pattern in cultured fibroblasts irradiated in vitro [12], patients were classified individually as either radiation-'sensitive' or radiation-'resistant'. Here, we attempt to verify the results of this predictive gene expression profile applied to the clinical findings in our cohort of Danish breast cancer patients.

Material and methods

The study cohort consisted of 214 women treated with BCT according to the Danish Breast Cancer cooperative Group (DBCG) 89 protocol, previously described in detail elsewhere [15]. All patients had been treated with BCS followed by whole breast

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(Received 23 June 2015; accepted 30 June 2015)

ISSN 0284-186X print/ISSN 1651-226X online
DOI: 10.3109/0284186X.2015.1069395

irradiation, 48 Gy in 24 fractions, and 177 patients were given a supplementary boost dose to the tumor bed. The women were distributed into three main groups based on the type of adjuvant treatment after BCT: no adjuvant treatment, chemotherapy, and antihormone therapy. Patients were seen at a clinical follow-up visit comprising an interview on post-operative course and comorbidity, and a physical examination.

The harvest of a 2×2 mm skin biopsy from the upper arm on the non-treated side was performed at the visit. In the laboratory fibroblast cultures were established (failed for four patients) and irradiated as described previously [16]. Briefly, fibroblasts from each patient were expanded and then divided into two groups; one group was irradiated in vitro (250 kV, 0.97 Gy/min) with 3.5 Gy on three consecutive days with 24 hours interval, whereas the other group was not irradiated. From both groups RNA was extracted and the gene induction patterns of nine genes (*CDC6*, *CXCL12*, *FAP*, *LMNB2*, *LUM*, *MXRA5*, *SOD2*, *SOD3*, and *WISP2*) was assessed using quantitative RT-PCR (failed in four patients). Patients were then classified individually using the mean and variance values for each of the nine genes in each cluster ('sensitive' and 'resistant') described in details elsewhere [13]. Classification was performed using the general principles described previously [17]. However, nine samples could not be assigned unanimously as either 'sensitive' or 'resistant' and the patients were excluded from further analyses. Fibrosis in the treated breast was the primary end-point and was estimated by palpation, using the non-treated breast as a reference. Fibrosis was scored on a four-point ordinal scale, according to the LENT-SOMA criteria [18], and then dichotomized into two groups: 'none to mild' and 'moderate to severe' fibrosis.

In our clinical study univariate logistic regression analyses were performed entering those variables with a biologically explicable relation to the development of fibrosis, followed by a multivariate analysis using stepwise backwards selection of variables with significant or borderline significant p-values [15]. This procedure was repeated with the inclusion of the gene expression profile. Information on patient's relevant comorbidity (diabetes mellitus, connective tissue disease etc.) did not affect the study results and were left out of the final analysis (data not shown). Data were analyzed using Stata version 14.

Results and discussion

Based on the patterns of induced gene expression in fibroblasts, 197 of 214 breast cancer patients could be classified. Patients from whom the culturing of fibroblasts or gene expression analysis failed (8), or whose patterns of gene expression fell in between the two subgroups (9) were excluded from further analysis. Of those with a classifiable gene expression pattern, 171 patients were identified with a 'sensitive' profile and 26 patients with a 'resistant' profile. Moderate to severe fibrosis was registered in 42/171 patients (25%) in the 'sensitive' subgroup compared to 2/26 patients (8%) in the 'resistant' subgroup. Table I shows the patient, tumor and treatment characteristics for the remaining cohort.

In the univariate analyses, breast size $\geq$ cup C, boost dose $>$ 10 Gy, and being a current smoker (vs. being none or previous smoker) were each found to a have significant association with the development of moderate to severe fibrosis. Variables identified as being independent predictors of developing moderate to severe fibrosis in the multivariate analysis were breast size $\geq$ cup C, CEF chemotherapy, boost dose $>$ 10 Gy and the gene expression profile (OR 7.32, CI 1.3–41.0). Table II shows the odds ratios for the selected variables in the uni- and multivariate logistic regression analyses.

The findings in our study validates that the gene expression profile, has predictive value for identifying patients susceptible to the development of radiation-induced fibrosis. The profile was originally developed in breast cancer patients [12], then subsequently validated in head and neck cancer patients [13] and here in breast cancer patients. The applicability in breast cancer patients treated with low doses of irradiation given as adjuvant treatment after surgery and in head and neck cancer patients treated with high doses of irradiation given as a primary curative treatment, illustrates the general nature of this profile.

Other strengths of this study are that the patients have been treated homogeneously both in regard to the RT and in regard to the adjuvant systemic treatment ensured by the national guidelines from the DBCG. All patients have been examined by the same clinician and patients were seen 7–20 years after their initial treatment with surgery and RT, ensuring that all possible normal tissue injury has been fully developed. A high number of patients were available for analyses compared to other similar studies. One possible limitation is that the subgroup of patients receiving a boost dose $>$ 10 Gy is small (10 patients), however, the finding that a high boost dose is associated with increased risk of fibrosis is supported by previous studies, including the Boost no Boost-trial [19]. Other limitations lie in the fact that it takes a minimum of six weeks from the harvesting of a skin biopsy to the answer from the gene expression analyses. The procedure is expensive, time consuming and so far not applicable to the daily clinic, where the decision on treatment has to be made on

Table I. Patient, tumor, and treatment characteristics.

	All		Only RT		Chemotherapy		Antihormone	
	n	%	n	%	n	%	n	%
Participants	197	100	68	35	63	32	66	33
Age at diagnosis (mean, range)	51.5	(25.9, 74.5)	51.2	(38.6, 68.2)	44.5	(25.9, 58.3)	58.5	(42.9, 74.5)
Follow-up length (median years, range)	12.3	(7.0, 20.2)	14.4	(9.4, 20.2)	12.4	(7.0, 19.8)	10.1	(7.6, 17.7)
Menopausal status								
Premenopausal	100	51	34	50	62	98.5	4	6
Postmenopausal	97	49	34	50	1	1.5	62	94
Chemotherapy								
All	68	32	0	0	63	100	0	0
CMF	28	13	0	0	27	43	0	0
CEF[a]	40	19	0	0	40	57	0[a]	0
Breast size								
$<$C	93	47	30	44	32	51	31	47
$>$C	104	53	38	56	31	49	35	53
Boost dose								
No boost	33	17	0	0	3	5	30	45
$\leq$10 Gy	156	79	65	96	57	90	34	52
$>$10 Gy	8	4	3	4	3	5	2	3
Fibrosis								
Moderate-Severe	44	22	10	15	18	29	16	24
None-Mild	153	78	58	85	45	72	50	76
Gene expression classifier								
Radiation-'resistant'	26	13	5	7	14	22	7	11
Radiation-'sensitive'	171	87	63	93	49	78	59	89

[a]10 patients in the chemotherapy group (CEF) also received antihormone treatment.

a day-to-day basis. To solve this we are investigating SNPs possibly linked to our gene expression profile, which by the use of a blood sample could more easily enable us to identify the same radiation-'resistant' and -'sensitive' patients.

Associations between gene expression levels in cultured fibroblasts and increased risk of radiation-induced fibrosis has been suggested in two previous studies, one using baseline gene expression levels [11] and one using induced levels after a single dose of 10 Gy [10]. None of the genes suggested in these studies overlap with the profile validated here. This may be explained by the fact that our profile is developed as a predictor of resistance to radiation-induced fibrosis, and has only been revealed after fractionated irradiation of the fibroblasts [12,14]. Also, although

Table II. Odds ratios for the independent predictors in the univariate and multivariate models.

	Univariate analysis		Multivariate analysis	
	OR (95% CI)	p-Value	OR (95% CI)	p-Value
Gene expression profile				
'Resistant'				
'Sensitive'	3.91 (0.89–17.23)	0.072	**7.32** (1.30–41.01)	**0.02**
Breast size				
$<$C				
$\geq$C	**3.00** (1.44–6.26)	**$<$0.01**	**3.54** (1.62–7.92)	**$<$0.01**
Adjuvant treatment				
None				
CMF	2.03 (0.68–6.05)	0.20	3.04 (0.90–10.30)	0.08
CEF	2.55 (0.96–6.77)	0.06	**3.81** (1.29–11.25)	**0.02**
Antihormone	1.86 (0.77–4.46)	0.17	2.24 (0.79–6.40)	0.13
Boost dose				
No boost				
$\leq$10 Gy	0.78 (0.32–1.88)	0.57	0.94 (0.32–2.81)	0.92
$>$10 Gy	**5.21** (1.01–26.80)	**0.048**	**12.22** (1.70–88.43)	**0.01**
Smoking status				
Previous/none				
Current	**2.28** (1.06–4.88)	**0.034**	1.89 (0.81–4.39)	0.14

Bold values refer to analyses (OR and p-values) with significant findings.

we are able to find patients sensitive to irradiation, this profile was truly developed to identify patients being resistant rather than sensitive to radiation-induced fibrosis.

In conclusion, we were able to validate the gene expression profile on our cohort of 197 Danish breast cancer patients. The gene expression profile could independently identify a subset of patients treated with BCS and whole breast irradiation, who demonstrated higher risk of developing moderate to severe fibrosis, using radiation-induced gene expression patterns from nine genes in fibroblast, irradiated in vitro. The general nature of this profile makes it potentially useful for the prediction of fibrosis in a wide selection of patients treated with irradiation.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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