NQO1 expression correlates inversely with NF κ B activation in human breast cancer

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Received: 9 May 2011 / Accepted: 3 June 2011 / Published online: 25 June 2011
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Abstract NQO1 participates in cellular defense against oxidative stress and regulates apoptosis via p53- and NF κ B-mediated pathways. We have previously found that homozygous missense variant NQO1*2 (rs1800566) predicts poor survival among breast cancer patients, particularly after anthracycline-based adjuvant chemotherapy. Here, we investigated NQO1 and NF κ B protein expression and global gene expression profiles in breast tumors with correlation to tumor characteristics and survival after adjuvant chemotherapy. We used immunohistochemical analysis of tissue microarrays to study NQO1 and NF κ B expression in two series of tumors: 1000 breast tumors unselected for treatment and 113 from a clinical trial comparing chemotherapy regimens after anthracycline

treatment in advanced breast cancer. We used gene expression arrays to define genes co-expressed with NQO1 and NF κ B. NQO1 and nuclear NF κ B were expressed in 83% and 11% of breast tumors, and correlated inversely ($P = 0.012$). NQO1 protein expression was associated with estrogen receptor (ER) expression ($P = 0.011$), whereas 34.5% of NF κ B-nuclear/activated tumors were ER negative ($P = 0.001$). NQO1 protein expression and NF κ B activation showed only trends, but no statistical significance for patient survival or outcome after anthracycline treatment. Gene expression analysis highlighted 193 genes that significantly correlated with both NQO1 and NF κ B in opposite directions, consistent with the expression patterns of the two proteins. Inverse correlation was found with genes related to oxidation/reduction, lipid biosynthesis and steroid metabolism, immune response, lymphocyte activation, Jak-STAT signaling and apoptosis. The inverse relationship between NQO1 protein expression and NF κ B

Electronic supplementary material The online version of this article (doi:10.1007/s10549-011-1629-5) contains supplementary material, which is available to authorized users.

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activation, underlined also by inverse patterns of association with ER and gene expression profiles of tumors, suggests that NQO1–NF κ B interaction in breast cancer is different from several other tissue types, possibly due to estrogen receptor signaling in breast cancer. Neither NQO1 nor NF κ B protein expression appear as significant prognostic or predictive markers in breast cancer.

Keywords Breast cancer · NQO1 · NF κ B · Chemotherapy · Metastasis

Introduction

To improve individualized therapy, novel predictive markers are needed that identify patients with expected poor breast cancer outcome or patients who benefit most from a given treatment. We have recently shown that NAD(P)H:quinone oxidoreductase 1 homozygous genotype, NQO1*2, predicts poor survival among breast cancer patients, particularly after anthracycline-based adjuvant chemotherapy and in p53-aberrant tumors [1]. This functionally important common missense variant (rs1800566, c.558C>T) destabilizes NQO1 and cells homozygous for the mutant allele are deficient in NQO1 activity. Whereas the wild-type NQO1 protein is stable in cells, the variant form of NQO1 is rapidly turned over via ubiquitination followed by proteasomal degradation [2–4]. NQO1 is expressed in various normal tissues including breast and elevated NQO1 expression was reported for breast, ovarian, pancreatic, thyroid, adrenal, colon, and corneal cancer [5–7].

NQO1 has multiple functions including protection against cytotoxicity and carcinogenicity of quinones and oxidative stress by catalyzing reduction and detoxification of quinone substrates [8, 9]. NQO1 plays a role in regulation of cell survival versus apoptosis through its interaction with p53 and links with the NF κ B pathway. NQO1 regulates the stability of p53, protecting it from 20S proteasomal degradation. Active NQO1 increases the basal level and induction of p53, potentiating apoptosis especially in response to oxidative stress [10–12]. Its role in NF κ B signalling is less clear. NF κ B is a transcriptional regulator of NQO1, and NQO1 in turn regulates NF κ B activation under certain conditions [13, 14]. While NF κ B activation usually triggers cellular pro-survival, pro-proliferation responses, it can also lead to cell death [15]. The role of NQO1 in this context also varies from antiapoptotic to proapoptotic, possibly depending on cell type and/or microenvironmental factors. In mouse models the loss of NQO1 is accompanied by impaired function of NF κ B, as TNF- α is unable to activate NF κ B in NQO1-deficient mice. Moreover, it has been demonstrated that treating NQO1-wild-type cells with dicoumarol, an inhibitor of

NQO1, abolishes TNF-induced NF κ B activation [13, 16]. We have also previously shown that NQO1 enhances TNF-induced apoptosis in human breast tumors [1], and a similar effect has been reported in breast carcinoma cells in vitro [17].

Nuclearfactor κ B (NF κ B) is a transcription factor that is mostly cytoplasmic and translocates to nucleus upon activation [18]. While the basal level of active NF κ B is minimal in most cell types, the constitutive activation of NF κ B has been observed in various tumor types including human breast cancer [19]. Emerging evidence indicates the involvement of constitutively active NF κ B in proliferation, angiogenesis, cell survival, metastasis, and apoptosis. For instance, in breast cancer cells, particularly with negative estrogen receptor status, NF κ B activity is constitutive and thought to protect tumor cells against apoptosis [20, 21]. Indeed, elevated NF κ B expression was suggested to associate with cancer cell resistance to radiation [22] and anthracycline-based chemotherapy [23]. Furthermore, while anthracycline-based treatment (doxorubicin) reportedly activates NF κ B in cancer cells [24], NF κ B signaling induced by doxorubicin was also implicated in reduced expression of NF κ B-dependent genes in cancer cells due to its instability in vivo [25].

Here, we analyzed NQO1 protein expression and NF κ B nuclear localization in an extensive series of sporadic and familial non-BRCA1/2 breast tumors and in a second series of primary breast tumors from patients in clinical trials comparing chemotherapy regimens after anthracycline treatment in advanced breast cancer, to investigate their correlation to clinical characteristics and breast cancer survival. Additionally, we used gene expression profiling to explore genes correlating with NQO1 and NF κ B expression in human breast tumors.

Patients and methods

NQO1 and NF κ B protein expression was analyzed in two series of invasive breast tumors. The first series included 884 tumors from unselected breast cancer patients and additional 542 familial cases. The unselected cases were ascertained at the Department of Oncology, Helsinki University Central Hospital, during the years 1997–1998 and 2000 as previously described [26, 27]. The additional 542 familial breast cancer cases were collected by a systematic screening at the Department of Oncology, Helsinki University Central Hospital or were ascertained through genetic counseling at the Department of Clinical Genetics. The total number of 1,238 invasive breast carcinomas were available for TMA as described [28], including 423 from cases without familial background of breast cancer and 815 from patients with family history. Of the familial cases,

480 patients have strong family history of breast cancer (three or more first or second degree relatives with breast or ovarian cancer in the family, including the proband), and 335 had two affected first-degree relatives (including the proband). BRCA1 and BRCA2 mutations had been excluded from the familial patient series as described [29–31].

Clinicopathological information on patients' characteristics, tumor size, nodal status, distant metastasis, estrogen and progesterone receptor status was collected from the pathology reports. A breast cancer pathologist (P.H.) re-reviewed all tumors for histological diagnosis and grade using the Scarff-Bloom-Richardson method, modified by Elston and Ellis [32]. Information on the tumor marker data used (p53, HER2, and ki-67) [33–36] can be found in the Supplementary Note.

Information on adjuvant treatment and distant metastases during the follow-up was collected from the patient records. The information on death due to breast cancer or other reason was obtained from the Finnish Cancer Registry. Survival was calculated as 10 years overall survival (10 years OS): the time from diagnosis to the date of death due to breast cancer or other reasons, and as 5 years survival from metastasis to death: the time from distant metastasis to the date of death. Detailed information on the survival analysis can be found in the Supplementary Note.

The second series consists of 283 breast cancer patients with distant metastasis who participated in a randomized multicenter trial comparing taxotere (docetaxel) with methotrexate-fluorouracil (MF) after failure of anthracycline treatment in advanced breast cancer [37]. From these, 113 primary breast tumors on tissue microarrays (TMA) were available for our analysis. TMAs were prepared as described [38]. Response evaluation was performed according to the WHO recommendations [39]. Treatment response, time to progression (TTP), and overall survival (OS) from start of anthracycline treatment preceding randomization were analyzed. For statistical analysis, clinical response was divided into two categories: response (complete or partial) and non-response (no change and progression). Overall survival (AOS) was calculated from the start of anthracycline treatment until death. Time to progression (ATTP) was calculated from the start of anthracycline treatment till disease progression. AOS and ATTP data were available for 70 and 55 tumors stained for NQO1 and NF κ B, respectively. Time to progression (TTP) on methotrexate-5-FU or docetaxel was measured from the date of randomization till disease progression. Of the 113 studied tumors, all were available and scored for NQO1, while 80 were available and scored for NF κ B.

This study was done with patients' informed consent and permissions from the Ethics Committee of the Helsinki University Central Hospital and the Ministry of Social

Affairs and Health in Finland. The study was performed according to reporting recommendations for tumor marker prognostic study (REMARK) [40].

Immunohistochemistry

TMAs were constructed as described [28], including four cores (diameter 0.6 mm) of the most representative area from each formalin-fixed and paraffin-embedded breast cancer specimen. For NQO1 analysis, TMA slides were stained with mouse monoclonal anti-human NQO1-antibody (Santa Cruz, diluted 1:500). For NF κ B analysis, the TMAs were stained with rabbit monoclonal anti-human NF κ B (ABCAM, diluted 1:1000). The secondary reagents were from Vector Laboratories (Burlingame, USA), and the chromogenic substrate enhancement step was performed as described, without nuclear counterstaining [41]. As only 0–1% of normal breast tissue or tumor cells were immunohistochemically positive for NQO1 in individuals with homozygous c.558C>T variant, we generally considered the breast tumors positive for NQO1 when 2% or more of the cancer cells were positive for NQO1 staining and negative when fewer than 2% were stained. For NF κ B immunohistochemistry, a 5% cut-off was reported for nuclear staining in prostate cancer [42]. Consistently, we observed only rare nuclear positivity in normal or benign breast tissue ($n = 30$), and therefore we also considered NF κ B as positive/activated in the breast carcinomas when 5% or more cancer cells showed nuclear staining and negative when fewer than 5% cells showed nuclear signal, or only cytoplasmic staining for NF κ B.

Microarray data analysis

Total RNA was extracted from 183 primary breast tumors. The samples were processed and hybridized to Illumina HumanHT-12 v3 Expression BeadChips, containing 24660 Entrez Gene entities, according to the manufacturer recommendations (<http://www.illumina.com>).

Microarray raw data were imported into R (<http://cran.r-project.org>) and processed by the methods included in the BioConductor facilities [43]. After quality control [44], the data were normalized using the quantile method [45] and the gene expression matrix was obtained by averaging the probes mapping to the same Entrez Gene IDs [46]. The Pearson's correlation of each gene expression profile with the expression of *NQO1* and *NF κ B1* was, respectively, calculated and the nominal *P* values were estimated. The genes with correlation *P* value <0.01 after Benjamini–Hochberg post hoc correction were further analyzed [47]. Functional annotation was performed on the *NQO1*-correlated and the *NF κ B1*-correlated gene lists using the DAVID annotation tools [48]. The categories

with Fisher's exact test P value <0.05 were considered to be significantly enriched.

Statistics

Statistical analyses were conducted in SPSS v15.0.1 for windows (SPSS, Inc.). The significance limit was set at 0.05. P values for evaluation of differences in NQO1 and/or NF κ B expression in varying tumor characteristics were calculated using Pearson's chi-squared tests or Fisher's exact test (for $n < 5$). All P values are two sided. Log-rank tests were used to assess the statistical significance of differences between Kaplan–Meier curves for survival analyses among all patients. Univariate Cox's regression analyses were used to estimate relative risk in various subgroups. To ascertain the effect of differential NQO1 protein expression on post-metastasis survival in the first series of patients, the beginning of follow-up time was set at date of diagnosed metastasis.

Results

We performed immunohistochemical analysis to study NQO1 and NF κ B expression for association with tumor characteristics and survival in two series of breast cancer patients. NQO1 and NF κ B staining results were obtained for 996 (80%) and 1030 (83%) tumors, respectively, from the first series of breast cancer patients. NQO1 expression

and NF κ B nuclear localization were seen in 83% and 11% of the tumors, respectively. There was no difference in the NQO1 expression or NF κ B nuclear localization by family history background, therefore, all tumors were subsequently combined in the same analyses. From the second series of patients with metastatic disease, NQO1 and NF κ B protein expression was obtained for 113 and 80 tumors, respectively. NQO1 was detected in 64% and NF κ B nuclear localization in 15% of the analyzable tumors, respectively. Representative examples of NQO1 and p65 NF κ B-staining pattern for normal human breast tissue and breast carcinomas with positive and negative NQO1 or NF κ B expression are shown in Fig. 1. The numbers of cases included in different analyses are shown in Supplementary Table 1.

Association between NQO1 protein expression and clinico-pathological data

There was an inverse correlation between NQO1 expression and NF κ B nuclear localization/activation ($P = 0.012$). Furthermore, negative NQO1 was associated with ER-negativity ($P = 0.011$) but not with any other tumor characteristics. As expected, NQO1 staining correlated with the NQO1*2 homozygous variant genotype ($P < 0.0001$). NQO1*2 homozygous (TT, Ser/Ser) cases were always NQO1 protein negative, whereas tumors from 171/264 (64%) of heterozygous patients and 541/573 (94%) of wild-type homozygous patients were positive for NQO1

Fig. 1 Immunohistochemical detection of NQO1 and NF κ B in human breast tumors. The images show NQO1-staining (top row) and p65 NF κ B-staining patterns (bottom row) for normal human breast tissue (a, d) and two breast carcinomas: one tumor with high

abundance of NQO1 and cytoplasmic/inactive p65 NF κ B (b and e, respectively), the other tumor lacking NQO1 and showing nuclear p65 NF κ B-staining indicative of NF κ B activation (c and f, respectively)

Table 1 Association of NQO1 protein expression with the clinicopathological features of the primary tumors in the first series of breast cancer patients with primary tumors

	<i>n</i> (%)			<i>P</i>
	Total (<i>n</i> = 996)	Positive exp. (<i>n</i> = 823)	Negative exp. (<i>n</i> = 173)	
Patients group				
Sporadic	357 (35.8)	295 (35.8)	62 (35.8)	0.884 Sporadic vs. familial
Large families	362 (36.3)	295 (35.8)	67 (38.7)	
Small families	277 (27.8)	233 (28.3)	44 (25.4)	
Tumor histology				
Ductal carcinoma	724 (72.6)	600 (72.9)	124 (71.6)	0.217 Ductal vs. lobular
Lobular carcinoma	165 (16.5)	130 (15.7)	35 (20.2)	
Medullary carcinoma	13 (1.3)	9 (1)	4 (2.3)	
Other	94 (9.4)	84 (10.2)	10 (5.7)	
Grade				
1	218 (21.5)	177 (21.8)	41 (23.9)	0.545, 0.304* 1 vs. 2 and 3
2	477 (47.2)	366 (45.2)	81 (47.3)	
3	315 (31.1)	266 (32.8)	49 (28.6)	
NA	16	14	2	
T				
1	564 (57.4)	468 (57.4)	96 (57.1)	0.932*
2	352 (35.8)	289 (35.5)	63 (37.5)	
3	34 (3.4)	33 (4)	1 (0.5)	
4	32 (3.2)	24 (2.9)	8 (4.7)	
NA	14	9	5	
N				
Negative	534 (54.4)	442 (54.3)	92 (54.7)	0.932
Positive	447 (45.5)	371 (45.6)	76 (45.2)	
NA	15	10	5	
M				
Negative	954 (96.5)	793 (96.9)	161 (94.7)	0.163
Positive	34 (3.4)	25 (3)	9 (5.3)	
NA	8	5	3	
ER status				
Negative	201(21.3)	155 (19.7)	46 (29.1)	0.011
Positive	742 (78.6)	630 (80.2)	112 (70.9)	
NA	53	38	15	
PgR status				
Negative	313 (33.2)	252 (32.1)	61 (38.6)	0.138
Positive	628 (66.7)	531 (67.8)	97 (61.3)	
NA	55	40	15	
p53				
Negative	772 (79.4)	637 (79.2)	135 (80.3)	0.834
Positive	200 (20.5)	167 (20.7)	33 (19.6)	
NA	24	19	5	
HER2				
Negative	846 (87.1)	695 (86.3)	151 (90.9)	0.126
Positive	125 (12.8)	110 (13.6)	15 (9)	
NA	25	18	7	
Ki-67				
Low	660 (66.7)	551 (67.3)	109 (63.7)	0.373

Table 1 continued

	<i>n</i> (%)			<i>P</i>
	Total (<i>n</i> = 996)	Positive exp. (<i>n</i> = 823)	Negative exp. (<i>n</i> = 173)	
High	329 (33.2)	267 (32.6)	62 (36.2)	<<0.001, <<0.001* TT vs. CC and CT
NA	7	5	2	
NQO1*2				
CC	573 (66.5)	541 (75.9)	32 (21.4)	
CT	264 (30.6)	171 (24)	93 (62.4)	
TT	24 (2.8)	0 (0)	24 (16.1)	0.012
NA	135	111	24	
NFκB				
Negative	863 (89.4)	724 (90.6)	139 (83.7)	0.012
Positive	102 (10.5)	75 (9.3)	27 (16.2)	
NA	31	24	7	

T tumor size, *N* nodal status, *M* primary metastasis, *ER* estrogen receptor, *PgR* progesterone receptor

* Chi-squared test for trend

Table 2 Association of NFκB protein expression with the clinicopathological features of the tumors in the first series of breast cancer patients with primary tumors

	<i>n</i> (%)			<i>P</i>
	Total (<i>n</i> = 1030)	Negative exp. (<i>n</i> = 913)	Positive exp. (<i>n</i> = 117)	
Patients group				0.696
Sporadic	360 (34.9)	321 (35.1)	39 (33.3)	
Large families	368 (35.7)	326 (35.7)	42 (35.8)	
Small families	302 (29.3)	266 (29.1)	36 (30.7)	0.017 Ductal vs. lobular
Tumor histology				
Ductal carcinoma	745 (72.3)	658 (72)	87 (74.3)	
Lobular carcinoma	167 (16.2)	158 (17.3)	9 (7.6)	
Medullary carcinoma	13 (1.2)	6 (0.6)	7 (5.9)	
Other	105 (10.1)	91 (9.9)	14 (11.9)	0.014, 0.135* 1 vs. 2 and 3
Grade				
1	232 (22.8)	194 (21.6)	38 (32.4)	
2	462 (45.5)	420 (46.8)	42 (35.8)	
3	320 (31.5)	283 (31.5)	37 (31.6)	
NA	16	16	0	0.930*
T				
1	585 (57.6)	517 (57.5)	68 (58.1)	
2	366 (36)	324 (36)	42 (35.8)	
3	32 (3.1)	29 (3.2)	3 (2.5)	
4	32 (3.1)	28 (3.1)	4 (3.4)	0.324
NA	15	15	0	
N				
Negative	551 (54.4)	482 (53.8)	69 (58.9)	1.000
Positive	461 (45.5)	413 (46.1)	48 (41)	
NA	18	18	0	
M				1.000
Negative	989 (96.7)	875 (96.6)	114 (97.4)	
Positive	33 (3.2)	30 (3.3)	3 (2.6)	

Table 2 continued

	<i>n</i> (%)			<i>P</i>
	Total (<i>n</i> = 1030)	Negative exp. (<i>n</i> = 913)	Positive exp. (<i>n</i> = 117)	
NA	8	8	0	
ER status				
Negative	207 (21.2)	170 (19.5)	37 (34.5)	0.001
Positive	768 (78.7)	698 (80.4)	70 (65.4)	
NA	55	45	10	
PgR status				
Negative	326 (33.5)	284 (32.8)	42 (39.2)	0.194
Positive	646 (66.4)	581 (67.1)	65 (60.7)	
NA	58	48	10	
p53				
Negative	798 (79.7)	709 (79.7)	89 (79.4)	0.901
Positive	203 (20.2)	180 (20.2)	23 (20.5)	
NA	29	24	5	
HER2				
Negative	865 (86.5)	762 (86.1)	103 (88.7)	0.563
Positive	135 (13.5)	122 (13.8)	13 (11.2)	
NA	30	29	1	
Ki-67				
Low	688 (67.2)	606 (66.8)	82 (70)	0.531
High	335 (32.7)	300 (33.1)	35 (29.9)	
NA	7	7	0	
NQO1*2				
CC	591 (66.2)	532 (66.8)	59 (61.4)	0.5
CT	278 (31.1)	242 (30.4)	36 (37.5)	
TT	23 (2.5)	22 (2.7)	1 (1)	
NA	138	117	21	
NQO1				
Negative	166 (17.2)	139 (16.1)	27 (26.4)	0.012
Positive	799 (82.7)	724 (83.8)	75 (73.5)	
NA	65	50	15	

T tumor size, *N* nodal status, *M* primary metastasis, *ER* estrogen receptor, *PgR* progesterone receptor

* Chi-squared test for trend

immunostaining. Results of NQO1 protein expression in relation to tumor characteristics, NFκB activation, and NQO1*2 mutation status in the first breast cancer series are summarized in Table 1.

In the second series of 113 patients treated with chemotherapy after metastatic disease, no significant association was observed between clinico-pathological data and NQO1 protein expression (Supplementary Table 2).

Association between NFκB activation and clinico-pathological data

Tumor characteristics according to NFκB immunohistochemistry in the first series are shown in Table 2. In

addition to the inverse correlation with NQO1 expression, nuclear NFκB was associated with ductal histology ($P = 0.017$), ER-negativity ($P = 0.001$) and lower grade ($P = 0.014$).

In the second series of patients treated with chemotherapy for metastatic disease, no significant association to NFκB expression was detected (Supplementary Table 3).

Survival analyses

Survival analysis by NQO1 immunostaining did not show significant differences among all patients or in subgroups stratified by adjuvant chemotherapy treatment (Fig. 2; Table 3). In the chemotherapy trial group, there were no

Fig. 2 Kaplan–Meier survival curves by NQO1 protein expression in the first series of patients with primary tumor. **a** 10-year overall survival after breast cancer diagnosis; 10-year overall survival among

individuals who received anthracycline treatment (**b**), non-anthracycline chemotherapy (**c**), no chemotherapy (**d**)

significant differences in treatment response, TTP or OS after first-line anthracycline treatment or after second-line docetaxel treatment (Supplementary Figure 1a–c; Table 4). However, patients with negative NQO1 expression had faster progression of the disease after second line methotrexate-fluorouracil treatment ($P = 0.03$, HR 1.99, 95% CI 1.04–3.78, Supplementary Figure 1d; Table 4). NF κ B protein localization/activation in tumors did not show any significant association with patient survival (Fig. 3; Supplementary Figure 2).

Although not statistically significant, NQO1 and NF κ B protein expression showed tendency for inverse survival after adjuvant anthracycline treatment (reduced survival for

patients with negative NQO1 or nuclear/activated NF κ B). Analogous non-significant survival tendency was found in both patient series for overall survival, and for survival from metastasis to death and for time to tumor progression after anthracycline treatment in the chemotherapy trial series (Figs. 2, 3; Tables 3, 4).

Microarray data analysis

In gene expression analysis, a total of 877 genes significantly correlated to NQO1 expression (293 positively and 584 negatively correlated) and 2,871 genes significantly correlated to NF κ B1 expression (1,635 positively and

Table 3 Univariate Cox's regression analysis by NQO1 and NFκB positive and negative protein expression for overall survival in the first series of breast cancer patients with primary tumors

Category	<i>n</i> (death)		<i>P</i>	HR	95% CI
	Positive exp.	Negative exp.			
10-year overall survival by NQO1 expression					
All patients	823 (169)	173 (39)	0.434	1.149	0.81–1.62
Adj-AC treated	120 (30)	29 (9)	0.377	1.401	0.66–2.96
Adj_Non_AC	157 (34)	29 (4)	0.339	0.603	0.21–1.70
No_Adj_Chemo	537 (101)	111 (25)	0.28	1.273	0.82–1.97
Overall survival from metastases to death (5 years), by NQO1 expression					
All patients	146 (102)	32 (23)	0.3	1.271	0.80–2.00
Adj_AC treated	34 (23)	11 (8)	0.201	1.693	0.75–3.79
Adj_Non_AC	33 (27)	3 (2)	0.402	0.541	0.12–2.27
No_Adj_Chemo	79 (52)	18 (13)	0.241	1.44	0.78–2.65
Category	<i>n</i> (death)		<i>P</i>	HR	95% CI
	Negative exp.	Positive exp.			
10-year overall survival by NFκB expression					
All patients	913 (197)	117 (23)	0.464	0.851	0.55–1.31
Adj_AC treated	143 (36)	11 (5)	0.113	2.135	0.83–5.45
Adj_Non_AC	164 (34)	22 (4)	0.657	0.791	0.28–2.22
No_Adj_Chemo	594 (122)	82 (13)	0.255	0.717	0.40–1.27
Overall survival from metastases to death (5 years), by NFκB expression					
All patients	168 (122)	16 (10)	0.57	0.83	0.43–1.58
Adj_AC treated	41 (28)	5 (4)	0.394	1.578	0.55–4.50
Adj_Non_AC	33 (28)	3 (1)	0.105	0.191	0.02–1.41
No_Adj_Chemo	94 (66)	8 (5)	0.959	0.976	0.39–2.42

Adj adjuvant, AC anthracycline, Chemo chemotherapy

1,236 negatively) (Supplementary Figure 3, Supplementary Tables 4 and 5). Interestingly, among genes correlating negatively with *NQO1* expression are *TNF* and 12 other genes related to the *TNF/NFκB* pathway as well as 4 genes related to Toll-like receptor (TLR) family. Altogether, 193 genes were significantly correlated both with *NQO1* and *NFκB1*. Only the gene *LIMA1* positively correlated both with *NQO1* and *NFκB1*, while all the other genes had inverse correlations with *NQO1* and *NFκB1*: 59 genes correlated positively with *NQO1* but negatively with *NFκB1*, while 133 genes correlated positively with *NFκB1* but negatively with *NQO1* (Supplementary Table 6). No genes showed concordant negative correlation both with *NQO1* and *NFκB1*.

Opposite trends of the *NQO1* and *NFκB1*-correlated genes were also observed when considering expression of functional gene families. The gene families of the endoplasmic reticulum, oxidation/reduction, lipid biosynthesis, and steroid metabolism were significantly over-represented both among the *NQO1*-positively correlated and *NFκB1*-negatively correlated genes. Furthermore, biological themes associated with immune response, lymphocyte

activation, Jak-STAT signaling, and apoptosis were significantly over-represented among the *NFκB1*-positively correlated and *NQO1*-negatively correlated genes (Fig. 4). Similar functional profiles were represented by the 193 *NQO1*- and *NFκB1*-commonly correlated genes (Supplementary Table 7).

Discussion

We have applied immunohistochemistry to study expression of *NQO1* and nuclear localization of *NFκB* in two series of human breast tumors, with correlation to tumor characteristics and survival and tumor progression after adjuvant chemotherapy. We found an inverse correlation between *NQO1* expression and *NFκB* nuclear localization, with correlation of *NQO1* expression with ER-positive tumors and *NFκB* nuclear expression with ER-negative tumors. The gene expression profiling corroborated and extended the opposing effects of *NQO1* and *NFκB* expression observed by the immunohistochemistry analysis. Interestingly, among genes which negatively correlated

Table 4 Univariate Cox's regression analysis by NQO1 and NFκB protein expression for overall survival and time to tumor progression in the chemotherapy trial series with metastatic disease

Category	<i>n</i> (event)		<i>P</i>	HR	95% CI
	Positive exp.	Negative exp.			
Overall survival and time to tumor progression by NQO1 expression					
First line of treatment after metastasis					
Anthracycline OS	52 (49)	18 (15)	0.674	1.134	0.63–2.03
Anthracycline TP	52 (52)	18 (18)	0.348	1.299	0.75–2.24
Second line of treatment after metastasis					
Taxotere TP	34 (30)	25 (20)	0.930	1.026	0.57–1.82
M-F TP	38 (36)	16 (16)	0.035	1.992	1.04–3.78
Category	<i>n</i> (event)		<i>P</i>	HR	95% CI
	Negative exp.	Positive exp.			
Overall survival and time to tumor progression by NFκB expression					
First line of treatment after metastasis					
Anthracycline OS	48 (43)	7 (7)	0.194	1.728	0.75–3.94
Anthracycline TP	48 (48)	7 (7)	0.098	2.041	0.87–4.75
Second line of treatment after metastasis					
Taxotere TP	37 (32)	7 (6)	0.583	1.285	0.52–3.13
M-F TP	31 (29)	5 (5)	0.169	1.993	0.74–5.32

Event is death for overall survival (OS) and tumor progression (TP) for other analyses

M-F methotrexate-fluorourasil

with NQO1 are TNF, TNF-related genes and TLR-related genes. Both families promote NFκB nuclear import [49]. *NQO1* gene expression correlated positively and *NFκB* gene expression negatively with genes implicated in cellular processes of oxidation/reduction, lipid biosynthesis, and steroid metabolism whereas the opposite correlation was found for biological themes associated with immune response, lymphocyte activation, Jak-STAT signaling and apoptosis, consistent with the published functions of NQO1 and NFκB. The association between NQO1 and ER expression was not unexpected, given the role of NQO1 in estrogen metabolism and the fact that NQO1 is in part transcriptionally regulated by ER [50]. Indeed, our gene expression analysis shows that several genes involved in steroid metabolism are co-upregulated with *NQO1* in breast tumors. On the other hand, NFκB nuclear expression was more common in ER-negative tumors, consistent with previous data [51]. ER and NFκB reportedly mutually repress each other, in addition to which there may be functional differences in NFκB signalling depending on ER status [52]. These findings are thus anticipated in view of previous literature. In contrast, the inverse relationship between NQO1 protein expression and NFκB activation is a novel finding, and somewhat unexpected given that they have been shown to be commonly co-activated in both normal and malignant cells, even upregulating each other [13, 14, 16, 53]. In melanoma, NQO1-mediated NFκB

activation (through stabilization of BCL3) may drive cell cycle progression and proliferation [54]. This disparity between breast cancer and other malignancies and tissues might reflect ER signalling in breast cancer. Given the documented mutual repression between ER and NFκB and the ability of ER to induce NQO1, it may be that the effects of ER override the mutual regulation between NQO1 and NFκB. Subgroup analyses of the association between NQO1 and NFκB in ER-positive and -negative cases separately (data not shown) unfortunately could not resolve this issue due to lack of statistical power especially in the smaller ER-negative group. In any case, it would appear that the NQO1–NFκB interaction in breast cancer differs from several other tissues, and functional elucidation of the underlying causal mechanism could provide insight into the biology of breast cancer and, potentially, its treatment.

In our previous study, we have found a strong association of the variant genotype NQO1*2 with poor prognosis of breast cancer patients, especially those treated with anthracycline-based chemotherapy. The homozygous patients showed increased mortality and reduced median survival time also after detection of metastasis suggesting resistance to treatment of the metastatic cells [1]. Here, we evaluated whether also NQO1 protein expression associates with patient survival in general and in treatment subgroups specifically. The results from this study suggest that NQO1 expression, or NFκB activation, do not appear to be

Fig. 3 Kaplan–Meier survival curves by NF κ B protein expression in the first series of patients with primary tumor. **a** 10-year overall survival after breast cancer diagnosis; 10-year overall survival among

individuals who received anthracycline treatment (**b**), non-anthracycline chemotherapy (**c**), no chemotherapy (**d**)

useful as prognostic or predictive markers in breast cancer. It is of note that while the NQO1*2 germ line genotype is strongly correlated with NQO1 protein expression, this correlation is not absolute. While all tumors from NQO1*2 homozygous patients stained negatively for NQO1, in line with previous reports [4, 55, 56], lack of NQO1 expression was also observed in 93/264 (35%) of the tumors from heterozygous carriers and 32/573 (5.5%) of wild-type homozygotes. In other words, tumors with negative NQO1 immunostaining but wild-type or heterozygous *NQO1* genotype are not directly analogous to NQO1*2 homozygous tumors. This could be due to a transient nature of NQO1 expression; lack of NQO1 immunostaining in a

given sample might not indicate that the cells are unable to express NQO1 in response to treatment, for example, but merely that the signalling event was not taking place when the sample was taken, or that the expression level is below detection threshold. Additionally, there does not appear to be strong selective pressure to lose the wild-type allele of NQO1 in breast tumors; in fact, the variant 609T allele was reportedly more commonly lost [57]. We observed no difference in genomic copy number of the region including *NQO1* gene (16q22) between tumors from the normal wild-type, heterozygous, or homozygous NQO1*2 variant carriers (data not shown) [58] further suggesting that the wild-type allele may be retained in the heterozygous carrier

NQO1	NFKB1	Gene Family
25	73	Endoplasmic reticulum
18	54	oxidoreductase
16	29	lipid biosynthesis
16	19	steroid metabolism
45	118	immune response
43	42	lymphocyte activation
16	38	Jak-STAT signaling pathway
32	23	apoptosis

Fig. 4 Functional representation of genes correlating with NQO1 and NFκB. The number of genes positively (*red*) and negatively correlated (*green*) and their functional annotation are shown

tumors, or not lost at least by genomic mechanisms. Given the lack of apparent LOH pressure, and the fact that NQO1 expression and NFκB activation were inversely correlated but not mutually exclusive, it is possible that the mutual upregulation between NQO1 and NFκB, as documented in other studies, could also occur in some breast tumors. This could have separate impact on survival and treatment response, and might confound survival analyses stratified by NQO1 immunohistochemistry alone. Discovering such subgroups in a survival analysis would however require a very large sample set.

Despite the lack of support for NQO1 (or NFκB) immunohistochemistry as prognostic or predictive markers, we observed that in the second series of patients treated with chemotherapy for metastatic disease, negative NQO1 immunostaining associated with faster disease progression after methotrexate-fluorouracil treatment following failure of anthracycline-based chemotherapy. This finding may suggest a pattern of differential association between NQO1 expression and response to various types of treatment, but lack of statistical power prevents a more granular analysis of the current material. We can speculate that NQO1*2 homozygotes are enriched in the anthracycline-resistant series, consistent with the higher frequency of tumors with negative NQO1 expression in this series, or that induction of NQO1 was lost by some other mechanism in advanced breast cancer due to treatment-related selective pressure. Very large studies are needed to further investigate association of NQO1 expression, preferably with available NQO1*2 genotype data, with the outcome

of combined or sequential treatment with different chemotherapy agents.

Acknowledgments We thank Drs. Karl von Smitten and Kirsimari Aaltonen and nurses Hanna Jäntti and Irja Erkkilä for help in collecting the patient samples and data. The Finnish Cancer Registry is gratefully acknowledged for the cancer diagnostic and follow-up data. This study has been funded by the Helsinki University Central Hospital Research Fund, the Sigrid Juselius Foundation, the Finnish Cancer Society, the Academy of Finland (132473), the Danish Cancer Society, the Danish National Research Foundation, the Czech Ministry of Education (MSM6198959216), the Czech Ministry of Health (NS10282-3/2009) and the European Commission (projects InflammCare, CZ.1.05/2.1.00/01.0030, and DDRResponse).

Conflict of Interest Licentia Ltd, representing Helsinki University Central Hospital and University of Helsinki, has filed PCT-patent application PCT/FI2007/050637 for NQO1, “Method for predicting the response to a therapy”.

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