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Efficacy of adjuvant chemotherapy according to Prion protein expression in patients with estrogen receptor-negative breast cancer

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Background: Prion protein (PrPc) has been previously reported to be associated with resistance to proapoptotic stimuli. We evaluated whether the expression of PrPc was associated with the resistance to adjuvant chemotherapy in patients with estrogen receptor (ER) -negative breast cancer.

Patients and methods: The expression of PrPc by primary tumors was assessed by immunohistochemistry in a series of 756 patients included in two randomized trials that compared anthracycline-based chemotherapy to no chemotherapy. The PrPc expression was correlated with ER expression and the benefit of adjuvant chemotherapy was assessed according to PrPc expression in patients with ER-negative tumors.

Results: Immunostaining analysis showed that PrPc was mainly expressed by myoepithelial cells in normal breast tissue. Tissue microarray analysis from 756 breast tumors showed that PrPc was associated with ER-negative breast cancer subsets ($P < 0.001$). Adjuvant chemotherapy was not associated with a significant risk reduction for death in patients with ER-negative/PrPc-positive disease [adjusted hazard ratio (HR) for death = 0.98, 95% confidence interval (CI) 0.45–2.1, $P = 0.95$], while it decreased the risk for death (HR = 0.39, 95% CI 0.2–0.74, $P = 0.004$) in patients with ER-negative/PrPc-negative tumors.

Conclusion: These data indicate that ER-negative/PrPc-negative phenotype is associated with a high sensitivity to adjuvant chemotherapy.

Key words: breast cancer, cellular prion protein, chemotherapy, clinical drug resistance, estrogen receptor negative

introduction

Breast cancer is a major cause of women morbidity and mortality in the world [1]. Anthracycline-based chemotherapy administered either before surgery (neo-adjuvant) or after surgery (adjuvant) for patient with stages I–III breast cancers improves survival rates [2] Nevertheless, the 10-year absolute benefit of adjuvant anthracycline-based chemotherapy is limited and ranged between 2% and 11%, indicating that most of the patients do not benefit from this treatment [3]. There is therefore a need to predict which patients get a benefit from adjuvant chemotherapy in order to better tailor adjuvant treatments and deliver the right drug at the right time and for optimal duration.

Several studies have been conducted with the aim to identify predictive factors of chemotherapy efficacy. Despite the lack of a global consensus, it has been suggested that hormone receptor status, tumor grade, and tumor cell proliferation could be predictive for the efficacy of chemotherapy. Poorly differentiated tumors with a high proliferation rate and without expression of hormone receptor are more chemosensitive and are associated with a higher percentage of pathologic complete response in neo-adjuvant setting. Sensitivity to anthracycline-based chemotherapy is also associated with Her2 overexpression [4], p53 status [5], and topoisomerase IIα amplification or deletion [6].

Recent studies have shown that estrogen receptor (ER) expression could split breast neoplasms in two highly distinct

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entities regarding gene expression profile [7]. Troester *et al.* [8], using tumor cell lines, have reported that the proteins involved in the response to anthracycline-based chemotherapy could differ according to cell lineage and ER status. From this, latter data have given rise the hypothesis that the biomarkers associated with efficacy of adjuvant chemotherapy could differ according to ER expression and cell lineage and that predictive biomarkers should be investigated separately in ER-negative and ER-positive disease [9].

Recently, using gene expression profile and *in vitro* cell model, we showed that ectopic expression of PrPc protects the mammary tumor cells from cell death induced by tumor necrosis factor (TNF) [10]. PrPc is a glycosylphosphatidylinositol-anchored protein, expressed by all known mammals, predominantly in the brain. Although PrPc is well-known for its implication in transmissible spongiform encephalopathy [11], several intriguing lines of evidence have emerged recently indicating that PrPc may function to protect cells from various kinds of internal or environmental stress. In this regard, PrPc overexpression rescues not only cultured neurons and yeast but also tumor cell lines from proapoptotic stimuli, including Bax expression [12–16], serum withdrawal [17], DNA damage [18], TNF [9], anisomycin [19], and anticancer drug treatments [20, 21].

The objective of this study was to evaluate whether PrPc expression by breast cancer correlates with resistance to chemotherapy. To address this question, we looked at the expression of PrPc protein in normal breast and breast cancer tissues and then evaluated the benefit of adjuvant chemotherapy according to PrPc expression in ER-negative breast cancer.

materials and methods

patients and treatment

PrPc expression was assessed by immunohistochemistry in normal breast tissue and subsequently in tumor samples from 823 patients included in two randomized trials that compared anthracycline-based chemotherapy to no chemotherapy. Results of these trials have been previously reported [22]. Considering results of immunostaining in normal tissues, we first hypothesized that PrPc was mainly expressed by ER-negative breast cancer subsets. Since results showed that PrPc was almost exclusively expressed in ER-negative disease, we next evaluated the benefit of adjuvant anthracycline-based chemotherapy in the ER-negative subset according to PrPc expression. In order to achieve this goal, we have analyzed the PrPc expression in 252 ER-negative tumors from the same two trials. In these trials, the chemotherapy regimen consisted in six courses of 5-fluorouracil 500 mg/m^2, doxorubicin, or epirubicin 50 mg/m^2, and cyclophosphamide 500 mg/m^2, administered i.v. on day 1. The interval between each chemotherapy course varied between 21 and 28 days according to hematological tolerance. After treatment completion, patients were seen every 6 months for the first 5 years, and yearly thereafter, with a yearly mammogram and a clinical examination at each visit.

tissue array and immunostaining

Primary tumors from 823 out of the 937 patients included in the two trials at Institut Gustave Roussy were used to build a tissue array. This tissue array contained three spots of each primary tumor. Each slide was stained with anti-PrPc (SAF 69, MAb) and anti-ER (clone 6F11, Novocastra) antibodies. Regarding PrPc staining, antigen retrieval was carried out with boiling in citrate buffer (10 mM/pH 7.0) for 20 min in a microwave oven (Microm/Micromed T/T) after deparaffinization and inactivation of endogenous peroxidase activity in methanol and H_2O_2. The slides were incubated with anti-PrPc monoclonal antibody that recognizes peptides 142–160 of human PrPc (1:550, SAF 69) for 1 h at room temperature. Anti-mouse peroxidase-labeled secondary antibody (DAKO ENVISION TM+system) and diaminobenzidine substrate were used to generate signal. For ER, staining was carried out according to manufacturer's recommendations.

Immunostainings were read by two investigators who were blinded for clinical files. PrPc staining was considered positive when a staining was observed in >10% of tumor cells. This cut-off was decided by analogy with ER and Her2 stainings. ER staining was considered positive when >10% of tumor cells were stained. When a discrepancy was observed between the three spots, the definitive score was the one observed in two spots. Normal breast epithelium and stromal cells served as an internal positive control. Omission of the primary antibody served as a negative control. Breast carcinoma MCF-7 cell line and its derivative clone served also as negative and positive control, respectively. When anti-PrPc monoclonal antibody was preincubated with peptides 142–160 of human PrPc (NH2-RYPNQVYYR-COOH, epytop), inhibition of immunohistochemistry was obtained, which confirmed the specificity of the antibody.

statistical analysis

In order to evaluate the efficacy of adjuvant chemotherapy according to PrPc expression in ER-negative subset, two sets of analyses were carried out. The prognostic value of adjuvant chemotherapy on overall survival (OS) was assessed in a multivariate analysis, both in PrPc-negative and PrPc-positive disease. The following variables were entered in the Cox model: age, tumor size, axillary node status, and tumor grade. These variables were chosen based on their previously reported prognostic values. The absolute benefit of adjuvant chemotherapy on OS was assessed using Kaplan–Meier curves both in PrPc-negative and PrPc-positive tumors. Survival curves were compared by a log-rank test.

results

PrPc expression is associated with ER-negative tumors

Since there is no report to date that PrPc is expressed in human breast tissue, we first analyzed PrPc expression in normal breast and breast cancer tissue by immunohistochemistry. Cytoplasmic expression of PrPc was seen in normal myoepithelial cells (Figure 1A). It is of note that some ER-negative breast cancer, notably the basal-like subtype, are hypothesized to arise from normal myoepithelial cell lineages cells. Using tissue microarrays, we next analyzed the PrPc expression according to ER status and clinical characteristics in a population of 823 breast cancer cases. Table 1 summarizes the characteristics of the assessable population (756 tumors). PrPc was expressed in 113 out of 756 evaluable tumors (15%). Expression was positive in all of the evaluable spots in 92 patients (81%), in two out of three spots in 16 patients (14%) and in one out of two spots in five patients (4%). PrPc was found to be expressed in 83 out of 252 ER-negative breast cancer (33%), while it was expressed in only 27 out of 499 (5%) ER-positive breast cancer ($P < 0.001$, chi square test). Representative stainings for PrPc expression are reported in

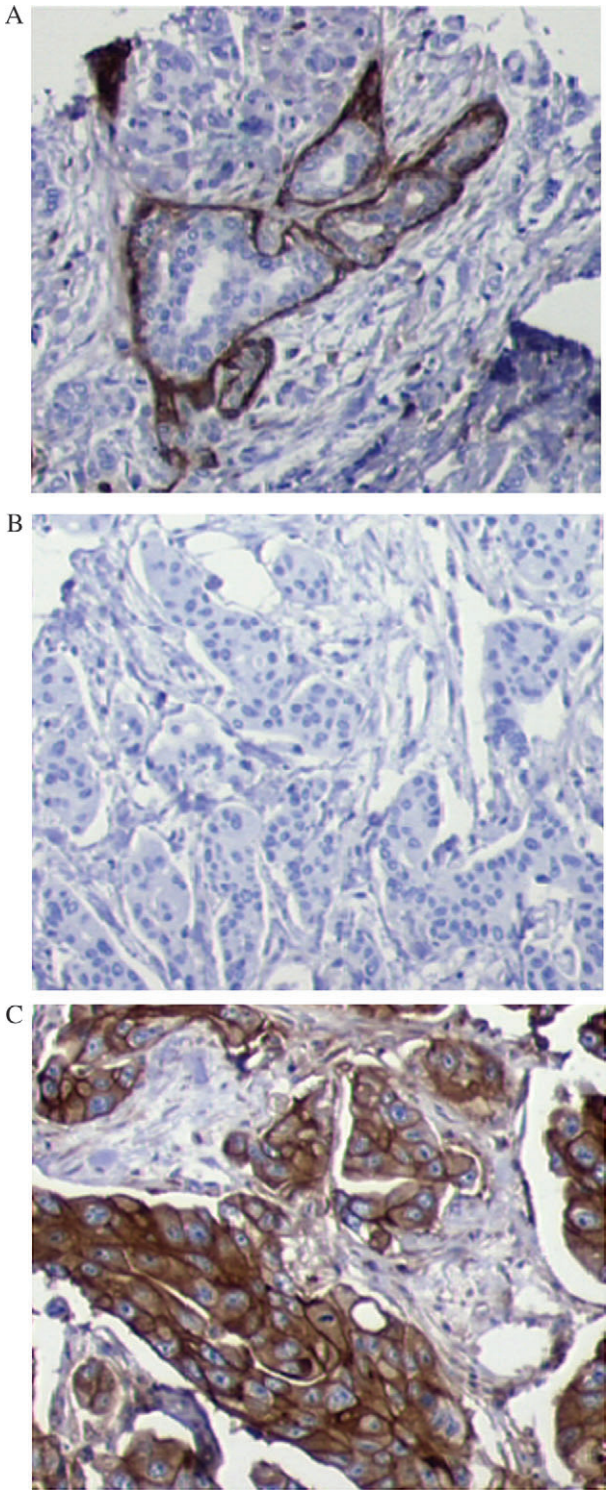

Figure 1. Prion protein (PrPc) expression by immunohistochemistry in tissues. PrPc expression in (A) normal breast myoepithelium, in (B) negative and (C) positive tumor.

Figure 1B (negative case) and Figure 1C (positive case). In addition to its relation with ER status, PrPc expression was also associated with high-grade tumors. Sixty-five percent of PrPc-positive tumors were high grade as compared with only 26% of PrPc-negative tumors. Based on these data indicating that PrPc is mostly expressed in ER-negative disease and on our previous data indicating that PrPc is involved in the resistance to cell death, we next focused on the association between PrPc expression and resistance to chemotherapy in patients with ER-negative tumors.

Table 1. Patient characteristics

	PrPc-positive tumors (n = 113)	PrPc-negative tumors (n = 643)
Median age (min–max)	55 (30–70)	57 (23–73)
Median tumor size (min–max)	20 (6–70)	20 (0–80)
Tumor grade		
I/II	39 (35%)	474 (74%)
III	72 (65%)	165 (26%)
Not assessable	2	4
Axillary node involvement	34 (30%)	285 (44%)
Estrogen receptor status[a]		
ER−	83 (75%)	169 (26%)
ER+	27 (25%)	472 (74%)
Not assessable	3	2

[a]Assessed by immunohistochemistry.
PrPc, prion protein.

PrPc expression and benefit of adjuvant anthracycline-based chemotherapy in ER-negative breast cancers

In order to address whether PrPc expression could be associated with resistance to anthracyclines in adjuvant setting, we analyzed the benefit of adjuvant chemotherapy according to PrPc expression in 252 patients with ER-negative disease. As reported in Figure 2, adjuvant chemotherapy significantly improved OS in patients with ER-negative/PrPc-negative disease (n = 169) (log-rank test, P = 0.006) (Figure 2A), while it was not associated with any survival improvement in patients with ER-negative/PrPc-positive tumors (n = 83) (log-rank test, P = 0.99) (Figure 2B). Ten-year OS rates were 79% [95% confidence interval (CI) 67% to 87%] and 64% (95% CI 52% to74%) in patients with ER-negative/PrPc-negative disease treated (n = 90) or not (n = 79) with adjuvant chemotherapy. On the other hand, 10-year OS rates were 68% (95% CI 52% to 81%) and 65% (95% CI 48% to 78%) in patients with ER-negative/PrPc-positive disease treated (n = 43) or not (n = 40) with adjuvant chemotherapy. We next analyzed the prognostic value of adjuvant chemotherapy by multivariate Cox analysis both in ER-negative/PrPc-negative and ER-negative/PrPc-positive tumors. As reported in Table 2, the performance of adjuvant chemotherapy was associated with a decreased hazard ratio (HR) for death in patients with ER-negative/PrPc-negative tumors (HR = 0.39, 95% CI 0.2–0.74, P = 0.004). On the other hand, the performance of adjuvant chemotherapy was not associated with a significant decreased HR for death in patients with ER-negative/PrPc-positive tumors (HR = 0.98, 95% CI 0.45–2.11, P = 0.95). Among the other variables, only the lymph node status was prognostic [HR = :1.1 (95%CI 1.01–1.2) and 1.15 (95%CI 1.1–1.22) in PrPc-negative and PrPc-positive tumors].

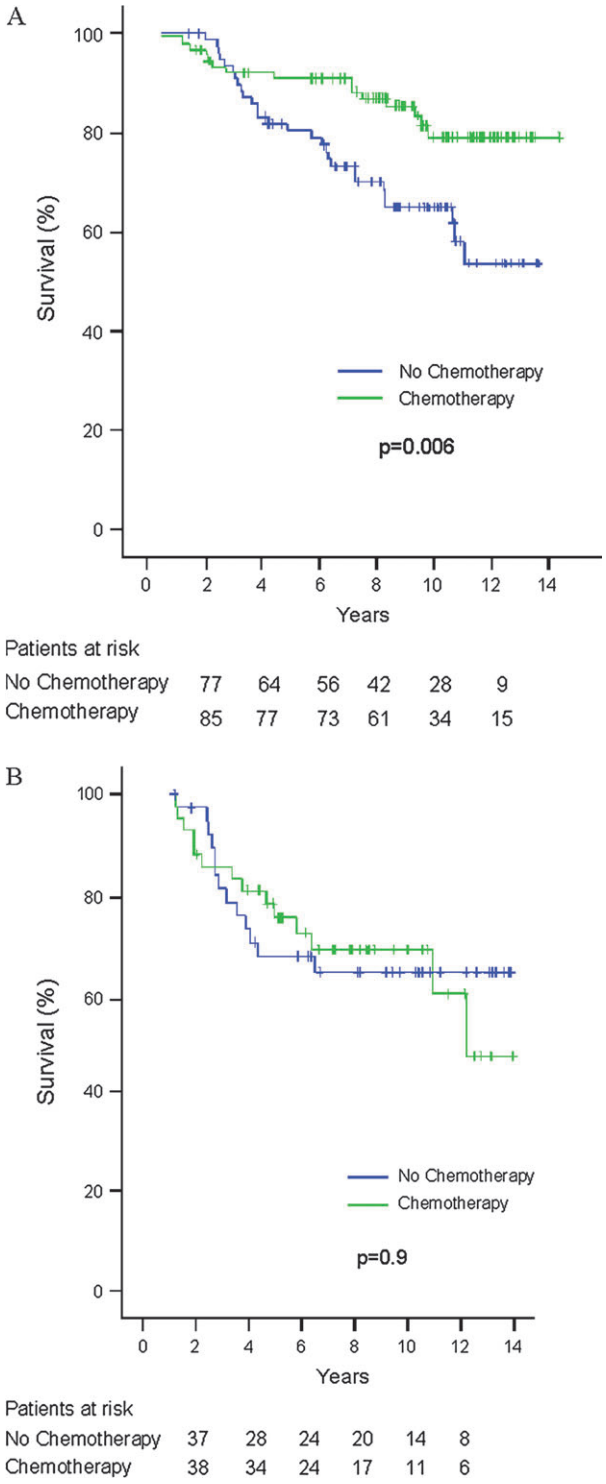

Figure 2. Absolute benefit of adjuvant chemotherapy in patients with ER-negative disease according to PrPc expression. Overall survival curves of patients with ER-negative breast cancer according to the performance of anthracycline-based chemotherapy in (A) PrPc-negative breast cancer and (B) PrPc-positive breast cancer. *P* values were calculated by using the log-rank test.

Overall, these data indicate that PrPc expression could be associated with resistance to anthracycline-based chemotherapy in patients with ER-negative disease.

discussion

The present study suggests that PrPc expression is associated with ER-negative disease and with a lower sensitivity to adjuvant chemotherapy in this patient subset. These data give rise to several questions: Are these data concordant with the current knowledge about PrPc function? Are these data concordant with other clinical studies? What are the potential perspectives opened by these data?

Although there is no report to date that PrPc is expressed in breast, several previous publications have reported the expression of PrPc by epithelium [26] and gastrointestinal tract [23]. More interestingly, recent data have reported that gastric adenocarcinoma could express PrPc [20, 21, 24]. Our data showed that normal breast could express PrPc. A striking finding from our study was the fact that only normal myoepithelial cells expressed PrPc. This finding is consistent with the data reported by Jones *et al.* [25] that classify *PRNP*, the gene that encodes for PrPc, in the top 50 myoepithelial-specific genes. In addition, our independent tissue microarray analysis from 756 independent patients confirm that PrPc was almost specific to ER-negative breast cancer subsets. Since PrPc was expressed in only 5% of ER-positive breast cancer, one could hypothesize that ER could modulate PrPc expression. Nevertheless, there is no data to date to support this hypothesis.

Several studies have reported that PrPc exhibit antiapoptotic properties in neurons [17, 26–28]. While the exact mechanism that leads to this resistance to apoptosis is unknown, there are some argument that PrPc interact with Bcl-2 [17, 29–31], Bax [14], and through a cAMP/PKA-dependent pathway [19]. Interestingly, PrPc has also been described to mediate Bcl-2 up-regulation and p53 down-regulation in gastric cancer cell lines [21], this latter phenomenon being well described to be associated with resistance to anthracycline-based chemotherapy.

In our study, PrPc expression was associated with a lower sensitivity to chemotherapy in ER-negative breast cancer. Although there is no other series published to date in this topic, there are some *in vitro* data that support this finding. We have previously shown that PrPc was associated with resistance to TNF-induced apoptosis [9]. Du *et al.* have reported that PrPc expression was induced in anthracycline-resistant gastric cancer cell lines. Interestingly, the authors reported that the mechanism that leads to drug resistance was actually more related to the induction of a multidrug resistance phenotype than to a resistance to apoptosis [20]. In their study, the PrPc inhibition by siRNA restored drug sensitivity *in vitro*.

Although our data indicated that PrPc could be predictive for the benefit of adjuvant chemotherapy in ER-negative disease, this study presents several limitations. The doses of chemotherapy in adjuvant setting were low (50 mg/m^2 for epirubicin) as compared with current standard. The number of patients was small and the finding that adjuvant chemotherapy

Table 2. Multivariate analysis of prognostic factors in ER-negative/PrPc-negative and in ER-negative/PrPc-positive tumors, respectively

	ER-negative/PrPc-negative tumors			ER-negative/PrPc-positive tumors		
	HR[a]	95% CI	*P*	HR	95% CI	*P*
Age	0.99	0.95–1.03	0.80	1.01	0.97–1.05	0.53
Tumor size	1.02	0.99–1.06	0.23	0.99	0.96–1.02	0.501
Lymph node status	1.1	1.01–1.2	0.02	1.15	1.1–1.22	<0.001
Tumor grade	0.75	0.33–1.7	0.49	1.61	0.85–3.05	0.143
Adjuvant chemotherapy	0.98	0.45–2.1	0.95	0.39	0.20–0.74	0.004

[a]Hazard ratio for death.

does not improve outcome in patients with ER-negative/PrPc-negative tumors is therefore not robust due to lack of statistical power in this subgroup. In addition, it was not possible to address the independency of PrPc as compared to other variables, including *TOP2A* gene amplification. Nevertheless, it must be emphasized that the study was done in a homogeneous subgroup of patients with highly sensitive tumors (i.e. those with ER-negative tumors) where no predictive biomarkers for chemotherapy efficacy has been validated.

The next step of this research project will aim at determining whether the high sensitivity to chemotherapy observed in ER-negative/PrPc-negative tumor is anthracycline specific or whether the same phenomenon could be observed with other drug regimen. If further studies show that PrPc is a drug-specific biomarker, then this parameter could be used to identify tumors with high sensitivity to anthracyclines. Since several other biomarkers have been reported to predict high sensitivity to anthracyclines (*TOP2A* amplification), this parameter could be included into a multimarker score. Finally, if further studies show that PrPc is not only a biomarker, but mediates by itself anthracyclines resistance, there would be a rationale to test PrPc inhibitors in combination with anthracyclines to enhance cell killing.

In conclusion, our study suggests that adjuvant chemotherapy could be highly effective in patients with ER-negative/PrPc-negative tumors, while its efficacy seems to be reduced in patients with ER-negative/PrPc-positive tumors. If confirmed by independent series, could open new avenues in the field of adjuvant therapies in ER-negative breast cancer.

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