Breast Cancer Research and Treatment **20**: 185–194, 1991.

Report

Prognostic significance of tumor phosphatidylcholine stearic acid level in breast carcinoma

Philippe Bougnoux,[1] Véronique Chajes,[1] Monique Lanson,[1] Kamel Hacene,[2] Gilles Body,[3] Charles Couet[4] and Olivier Le Floch[5]

[1] *Laboratoire de Biologie des Tumeurs, CNRS URA 1334 et Faculté de Médecine, 37032 Tours;* [2] *Dept. de Biostatistiques, Centre René-Huguenin, 92211 Saint-Cloud;* [3] *Clinique Gynécologique et Obstétricale, CHU, 37044 Tours;* [4] *Laboratoire de Nutrition, CHU, 37044 Tours;* [5] *Clinique d'Oncologie et Radiothérapie, CHU, 37044 Tours, France*

Key words: breast cancer, metastasis, phospholipids, fatty acids, stearic acid

Abstract

The involvement of lipid enzymes in the action of oncogenes at the cell membrane level has suggested that membrane lipids could play a role in modulating the growth of tumors. We previously found that breast cancer patients with a low level of polyunsaturated fatty acids in their primary tumor's phosphatidylethanolamine had a high risk of early occurrence of visceral metastasis. In the present study, we prospectively examined whether fatty acid composition of tumor membrane phosphatidylcholine had a prognostic significance in a series of 63 patients with a localized presentation of breast cancer. Membrane phospholipids were extracted from the carcinoma tissue obtained at the time of surgery, phosphatidylcholine was purified, and its fatty acids were analyzed by capillary gas chromatography. During the follow-up period, 20 patients developed metastasis. In these patients, the proportion of stearic acid containing phosphatidylcholine was significantly lower than it was in the tumors of the 43 patients who remained metastasis-free. Multivariate analysis according to Cox showed that low stearic acid level in tumor phosphatidylcholine and high mitotic index were independently predictive of subsequent metastasis. The predictive value of stearic acid level on metastasis risk was higher in node-positive patients than in node-negative patients, allowing individualization of a subgroup of low stearic acid level, node-positive patients with very poor prognosis. We concluded that stearic acid level in tumor membrane phosphatidylcholine is an independent intra-tumor marker of breast cancer prognosis. This finding is new evidence that tumor's structural lipids are linked to the growth of breast cancer.

Abbreviations: PC – Phosphatidylcholine, PE – Phosphatidylethanolamine, TLC – Thin-Layer Chromatography, CV – Coefficient of Variation, SD – Standard Deviation

Introduction

Compelling evidence suggests that membrane lipids are involved in basic cellular processes controlling cell proliferation [1]. Besides their structural role as part of the lipid bilayer, besides their metabolic role as reserve for precursors of biologically active compounds, they are specifically involved in

Address for offprints: P. Bougnoux, Laboratoire de Biologie des Tumeurs, CNRS URA 1334 et Faculté de Médecine, 2 bis Bvd Tonnellé, F-37032 Tours cedex, France

the regulation of protein kinase C activity, a key enzyme in the transmission of mitotic signals from the cell surface, through generation of diacylglycerol or through its interaction with phosphatidylserine [2]. Lipid enzymes have been recognized as effectors of pathways activated in growth signal transduction [3], and some of these enzymes may become targets for the action of oncogene products involved in cell transformation and acting at the cell membranes [4]. Altered lipid metabolism has been shown in *ras*-transformed cells [5]. Moreover, cell transformation induced by an activated *ras* oncogene has been reported to be modulated by membrane-incorporated fatty acids [6], suggesting in turn that membrane lipids might have a role in modulating the growth of tumors.

We have therefore examined the role of membrane lipid composition of the primary tumor in the evolution of breast cancer. Because of animal data emphasizing the metastasis-promoting role of polyunsaturated fatty acids [7], we initially investigated the fatty acid composition of phosphatidylethanolamine, a phospholipid known to be enriched in polyunsaturated fatty acids. We reported the existence of an association between a low level in n-6 polyunsaturated fatty acids and early occurrence of metastasis, suggesting a role for tumor membrane fatty acid composition in the clinical behavior of a human cancer [8]. However, we did not examine the fatty acid composition of other phospholipid classes in this previous report.

Phosphatidylcholine is a lipid class that should be more closely investigated in breast cancer. Phosphatidylcholine (PC) has a diversity of forms and functions. Upon phospholipase A2 activation its ether-linked form can supply several precursors of lipid mediators of inflammation: arachidonic acid, the precursor of eicosanoids [9], and lyso-paf. Lyso-paf, the product of sn-2 deacylation, is the precursor of paf, a biologically active lipid which has been found in breast tumor tissues [10]. Secondly, PC has been recently recognized as a link in the chain of events that occur during signal transduction, different from that described with phosphatidylinositol-2-phosphate [11, 12]. PC biosynthesis has been shown to be stimulated by growth factors such as the insulin-like growth factors or epidermal growth factor [11], and a receptor for these factors has been found in breast carcinoma [13, 14] and reported to be of prognostic significance [13].

The potential implications of PC in the control of tumor growth prompted us to investigate the fatty acid composition of membrane PC in breast tumors in relation to the clinical outcome of the disease. We prospectively carried out the study in a series of 63 patients undergoing surgery for a localized presentation of breast cancer, and we compared the spectrum of fatty acids with the development of systemic metastasis. We report here on the identification of a link between a low level of stearic acid in tumor phosphatidylcholine and poor prognosis, and on the independence of this tumor membrane marker of subsequent metastasis from other prognostic factors of breast cancer.

Methods

Patient population

Sixty-three previously untreated women with a localized presentation of invasive breast carcinoma were entered into the study when tumor tissue was available, and when adjuvant treatment and follow-up was performed in the University Hospital of Tours. All patients were initially examined by the same team of physicians, including radiation oncologists, gynecologists, and medical oncologists, using standardized rules for staging [15]. All patients underwent surgery in the Department of Gynecologic Oncology. Patients' characteristics are in Table 1. The age at the time of diagnosis ranged from 27 to 81 (median age 55). Mammography, chest X ray, bone scan, and ultrasonography of the liver, hemogram, tumor seric markers, and liver function tests were systematically performed. There was no detectable visceral metastasis at the time of surgery. The first therapeutic step was either mastectomy or lumpectomy according to the tumor and breast size, and to tumor localization within the breast, along with concommitant axillary dissection. Radiation therapy was delivered in the chest wall after mastectomy, or within the whole breast after lumpectomy with boost in the tumor bed, and in the internal

mammary, axillary, and subclavicular nodes in axillary node-positive patients. Internal mammary nodes were also irradiated when the tumor was in the internal or the central part of the breast. Adjuvant chemotherapy (mitoxantrone 12 mg/m^2, 5-fluorouracil 750 mg/m^2, and cyclophosphamide 600 mg/m^2 every 3 weeks for 9 cycles) was given in premenopausal patients with axillary node extension or with high histoprognostic grade and steroid receptor negative tumor, and in postmenopausal patients with node-positive, receptor-negative tumor, when possible. Hormonal therapy (tamoxifen 30 mg/day) was given in receptor-positive, postmenopausal patients for 2 years. Follow-up was carried out at regular intervals (every 4 months during the first year, then every six months). Investigations were performed when indicated, in order to unambiguously assess the presence of systemic metastasis.

Tissue examination and pathologic criteria

Carcinoma tissue was processed for pathology and stained according to accepted pathological procedures (hematoxylin-eosin-safran). Lymph nodes obtained from axillary dissection (mean 16, extremes 5–28) were examined for metastasis. The minimal number of nodes examined in lymph node negative patients (N–) was 8. Histopathologic types were defined according to the World Health Organization classification. All patients had an invasive breast carcinoma, which was predominantly of the ductal type in 57 patients, while 3 patients had a pure lobular type. In 3 patients, both types were detectable within the same tumor, or type was not determined. Histoprognostic grade was based on the evaluation of grading parameters (mitosis, tubular formation, and nuclear pleomorphism) and was provided in 3 classes when applicable. Mitotic index was determined by the method of Bloom and Richardson [16]. Specifically, it was evaluated by the maximum number of mitoses seen in a single field at 400X magnification, after examining 10 successive fields. Grade I was given when a maximum of one mitosis was seen in any one field, III when three or more mitoses were seen in any one field, and II in the intermediate situation. Steroid receptor level was measured on the tumor's cytosol using a standardized immuno-enzymo-assay [17].

Tissue preparation, lipid extraction and separation

Tissue specimens were obtained from the primary tumor during surgery. After excision of visible fat, samples were washed in saline and frozen in liquid nitrogen. At the time of processing, tumor samples were pulverized in liquid nitrogen and homogenized (Ultra-Turrax) at 4°C in a 0.15 M phosphate buffer pH 7.4. Lipids were extracted from the membrane-enriched pellet obtained after centrifugation (60,000 g, 1 h) at 4°C. The chloroform phase was filtered and phospholipids were separated by 2-dimensional TLC on Silica Gel G plates (LK-5, Whatman, Clifton, NJ) as already described [18]. Lipids were visualized with 0.2% 2',7'-dichlorofluorescein in ethanol, and the gel was scraped into screw-capped glass tubes containing 25 µg of 17: 0 fatty acid as an internal standard. Every class of phospholipid was extracted and transmethylated with 2 ml of 14% boron trifluoride in methanol at 105°C for 90 min [19]. Fatty acid methyl esters were extracted into hexane (HPLC grade, Carlo-Erba, Milano, Italy), concentrated to dryness under nitrogen, and injected through an on-column injector at 65°C into a gas chromatograph (Carlo Erba Instruments, Milano, Italy) operated at column and detector temperatures of 190 to 225 and 250°C. Helium was used as carrier gas at a flow rate of 1 ml/min with N_2 as make-up gas for the flame ionization detector. A 25 m × 0.32 mm I.D. fused silica capillary column with a 0.25 µm film thickness of Supelcowax10 (Supelco Inc., Bellefonte, PA) was used for the separation of methyl esters and dimethylacetals. Fatty acid methyl ester peaks were identified by their retention times based on an analysis of commercial standards (Sigma Chem. Co., St. Louis MO; Supelco; and Nu Check Prep, Elysian MN, USA). Quantification was made by comparison of the peak areas of each fatty acid methyl ester with that of the internal standard, and computed with an integrator (SP 4270, Spectraphysics, San Jose CA, USA). Results were

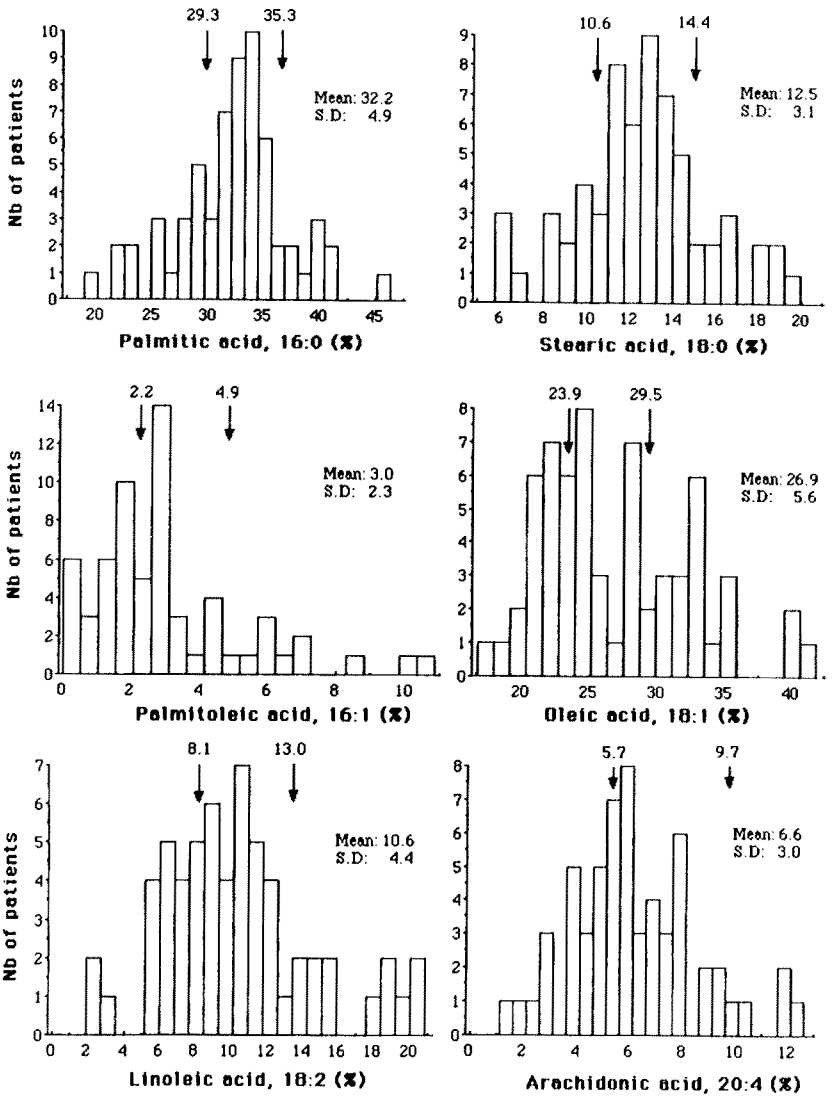

Fig. 1. Distribution of patients according to tumor phosphatidylcholine fatty acid levels. Membrane lipids were extracted from tumor tissue, phospholipids purified, and fatty acids analyzed as methyl esters by capillary gas chromatography. Mean value and SD on the whole population (n = 63) are shown for the 6 main fatty acids on each panel. Other fatty acids included saturates (14 : 0 and 20 : 0, accounting for less than 2%) and dihomogammalinolenic acid (20 : 3, mean 1.9%, range 0.1–8.4%). n-3 fatty acids were less than 1% of PC fatty acids. Unidentified peaks accounted for less than 6.8% of total area. Cut-off values are indicated for each fatty acid with arrows.

expressed as % of total area. Phospholipid phosphate was assayed on the lower phase remaining after transmethylation and hexane extraction as already described [18].

Reproducibility of results was examined in three different ways. 1) Repeatability of fatty acid quantification was assessed (n= 30). Intra-assay coefficient of variation (CV) ranged from 0.7% for large peaks such as palmitic acid to 2.6% for small peaks such as palmitoleic acid, with intermediate values for other peaks [20]. 2) Reproducibility was then examined by performing separately three times the whole procedure from the same tumor fragment. Inter-assay CV was 2.3 for palmitic acid, and 0.6% for stearic acid. 3) Since tumor tissues are heterogeneous, separate analysis were carried out on fragments sampled at different sites within the same tumor. Three tumors were large enough to allow 4, 4, and 5

separate fragments to be analyzed, and CV ranged from 1.2 to 8.5% for palmitic acid, and from 4.5 to 5.2% for stearic acid, according to tumors. However, since tumor fragments used for measuring PC fatty acid composition represented about half of the tumors in the present study, a potential heterogeneity of fatty acid content was averaged.

Statistical methods

Cut-off values for each of the fatty acids from tumor phosphatidylcholine and for the clinical size were determined by the K-means clustering method [21] which separates patients into clusters so that the within-cluster sum of squares is minimized. Chi-square tests were used to assess the association between fatty acid levels and conventional prognostic variables.

Metastasis-free and overall survival rates were calculated by the Kaplan-Meier method [22], and compared by the Logrank test [23]. The stepwise proportional-hazards regression model [24] was used to determine the significance of fatty acid levels on metastasis-free and overall survivals, with simultaneous adjustment for the effect of classical prognostic factors.

Table 1. Univariate analysis of clinical prognostic factors

Prognostic factor	Patients		Metastasis-free survival				Overall survival			
	(n)	(%)	No. of observed metastases (O)	No. of expected metastases (E)	O/E	p*	No. of observed deaths (O)	No. of expected deaths (E)	O/E	p*
Tumor size (mm)						0.10				0.63
≤25	14	22.2	6	4.4	1.37		2	2.3	0.88	
26–55	40	63.5	10	12.8	0.78		6	6.7	0.89	
>55	7	11.1	4	1.8	2.24		2	1.0	1.94	
na	2	3.2								
Nodal status						0.80				0.35
negative	28	44.4	9	9.0	1.00		3	4.5	0.67	
positive	35	55.5	11	10.5	1.05		7	5.5	1.27	
Histoprognostic grade						0.10				0.03
I (low)	2	3.2	0	0.8	0		0	0.4	0	
II (moderate)	39	61.9	10	13.3	0.75		3	6.6	0.46	
III (high)	21	33.3	10	6.0	1.68		7	3.1	2.27	
na	1	1.6								
Menopausal status						0.80				0.50
premenopausal	24	38.1	7	7.6	0.93		3	4.0	0.76	
postmenopausal	38	60.3	13	12.5	1.04		7	6.0	1.16	
perimenopausal	1	1.6								
Estrogen receptor						0.08				0.81
≤10	10	15.9	6	2.7	2.21		2	1.3	1.56	
>10	47	74.6	13	15.1	0.86		5	7.7	0.65	
nd	6	9.5	1	0.5	0.45		1	1.0	0.98	
Progesterone receptor						0.26				0.40
≤10	11	17.5	5	2.6	1.92		3	1.6	1.86	
>10	49	77.8	13	15.7	0.83		7	7.9	0.89	
nd	3	4.8	2	1.7	1.14		0	0.5	0	

*Logrank p-value; na: not applicable; nd: not done.

Results

Lipid composition of breast carcinoma

We carried out the fatty acid analysis of membrane phospholipids in tumor tissues obtained from breast cancer patients at the time of initial surgery. Phospholipids, assessed as lipid phosphorus, accounted for 4.1 μmol/g of tumor tissue (mean value; range 0.9 to 11.8). Phosphatidylcholine (PC) accounted for 40.7%± 6.9% (mean value ±SD) of total lipid phosphate. There were large differences in tumor membrane PC fatty acid composition among patients; mean levels and frequency distribution are presented for the main individual fatty acids in Fig. 1.

Relationship between PC fatty acid levels and prognostic factors

Several clinical and pathological characteristics have been identified to be related to the risk of metastasis [15]. Therefore, a link between prognostic features of the disease and fatty acid composition of tumor phospholipids was researched. For this purpose, two cut-off values were set for each fatty acid (Fig. 1) in a prognostic-independent manner from the whole distribution of results obtained from all tumor tissues available. This led for each fatty acid to the individualization of 3 populations of patients. In each population, the distribution of patients was examined according to tumor size (stratified in 3 groups, Table 1), menopausal status (pre-, peri-, or

Table 2. Fatty acid levels in tumor phosphatidylcholine and prognosis

Fatty acid	Definition levels (%)	Patients	Metastasis-free survival				Overall survival			
		(n)	No. of observed metastases (O)	No. of expected metastases (E)	O/E	p*	No. of observed deaths (O)	No. of expected deaths (E)	O/E	p*
16:0	Palmitic acid					0.025				0.0006
	≤29.3	17	4	5.7	0.70		1	3.1	0.32	
	>29.3–≤35.5	33	9	11.4	0.79		3	5.3	0.56	
	>35.5	13	7	2.8	2.47		6	1.6	3.81	
18:0	Stearic acid					0.034				0.0005
	≤10.6	16	8	3.8	2.13		7	2.1	3.37	
	>10.6–≤14.4	32	10	11.4	0.88		3	5.2	0.57	
	>14.4	15	2	4.9	0.41		0	2.7	0	
16:1	Palmitoleic acid					0.08				0.18
	≤2.2	28	5	9.3	0.54		2	4.8	0.42	
	>2.2–≤4.9	25	12	7.6	1.58		6	3.6	1.67	
	>4.9	10	3	3.2	0.95		2	1.6	1.26	
18:1	Oleic acid					0.10				0.047
	≤23.9	24	6	9.1	0.66		0	3.8	0	
	>23.9–≤29.5	20	9	5.0	1.79		5	3.1	1.62	
	>29.5	19	5	5.9	0.85		5	3.1	1.60	
18:2	Linoleic acid					0.95				0.48
	≤8.1	21	6	5.9	1.02		3	3.4	0.88	
	>8.1–≤13.0	27	9	8.6	1.05		6	4.3	1.40	
	>13.0	15	5	5.6	0.90		1	2.3	0.43	
20:4	Arachidonic acid					0.76				0.047
	≤5.7	28	10	8.9	1.13		4	4.2	1.89	
	>5.7–≤9.7	27	8	8.1	0.99		6	4.3	0.47	
	>9.7	8	2	3.1	0.65		0	1.5	0	

*Logrank p-value.

post-menopausal), lymph-node status (0 vs. 1 or more positive axillary nodes). Clinical size of the tumor was inversely associated with levels of stearic and linoleic acids ($p = 0.012$ and $p < 0.0001$, respectively). There was a low level of arachidonate in post-menopausal patients ($p = 0.035$). No significant association was found between axillary lymph node status and any of the fatty acid levels in PC. Among histopathologic features of the tumors, histoprognostic grade was inversely associated with levels of stearic acid in the PC of the tumor ($p = 0.03$). Positive estrogen receptor status (>10 fmol/mg protein) of tumors was associated with low palmitoleic acid level ($p = 0.004$).

Univariate analysis

Twenty out of the 63 patients developed visceral metastases during the follow-up period (mean follow-up, 30 months), and ten patients died as a consequence of their disease. Results according to occurrence of such events are presented in Tables 1 and 2. Metastasis-free and overall survival rates were calculated and compared for each conventional prognostic factor (Table 1), and for each fatty acid (Table 2). Although the proportion of patients who subsequently developed metastasis was greater in the group with high histoprognostic grade than in the low or moderate grades, or in the group of patients estrogen receptor negative than in the positive group, the differences did not reach statistical significance (Table 1). In contrast, low stearic and high palmitic acid levels were predictive factors of either metastasis or death (Table 2). Low levels of arachidonic acid or high levels of oleic acid were also slightly associated with risk of death.

Table 3. Multivariate analysis of prognostic factors

Significant variable	Regression coefficient	S.E.	Stepwise p-value
Stearic acid in PC	−0.244	0.087	0.002
Mitotic index	1.232	0.512	0.012

Global chi-square: 7.34 with 2 df ($p = 0.025$).

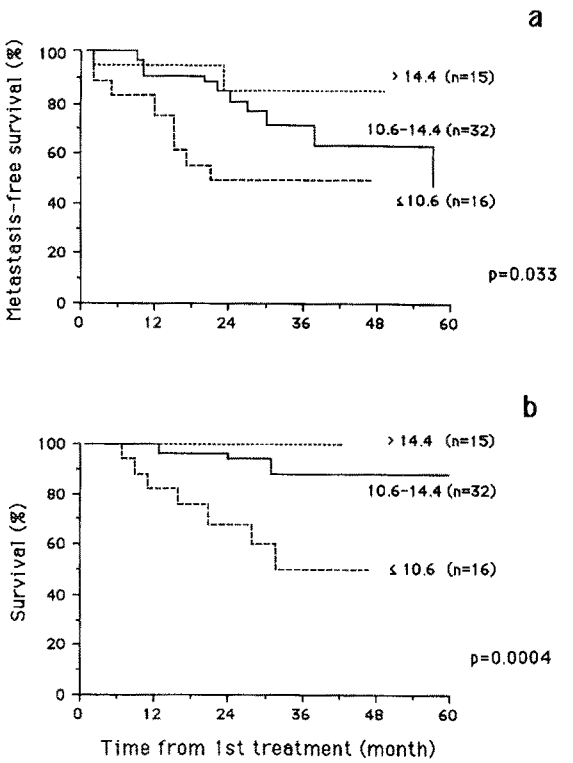

Fig. 2. Stearic acid content of tumor phosphatidylcholine and prognosis. Panel a shows metastasis-free survival and panel b overall survival as a function of stearic acid levels in tumor PC. Two thresholds of stearate levels were set, at 14.4% and 10.6% of total fatty acids in PC, defining 3 groups of patients with high (....), medium (——), or low (---) levels of stearic acid. Metastasis occured in 20 patients, within 1 to 59 months (average 17 months). Time to follow-up averaged 31 months for patients who remained metastasis-free.

Multivariate analysis

Since several fatty acid levels seemed associated with conventional prognostic factors on the one hand, and also with metastasis occurrence or death on the other, there was a need to determine whether one of them was predictive of prognosis independently from the other factors, or whether there were confounding factors. Multivariate analysis according to the Cox model showed that when all conventional prognostic factors of breast cancer and all individual fatty acid levels in PC were taken into account, only stearic acid level along with mitotic index appeared to be independent predictive factors of subsequent metastasis (Table 3). When mitotic in-

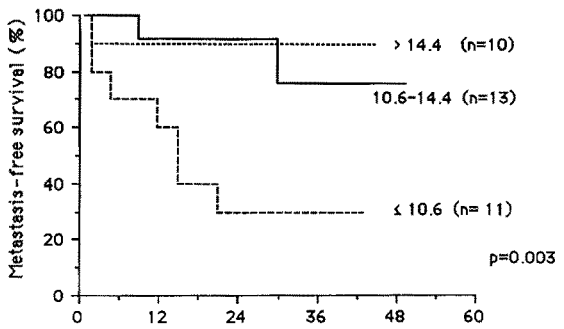

Fig. 3. Patients with positive axillary lymph nodes (N+ patients); probability of remaining metastasis-free according to stearic acid content of tumor phosphatidylcholine.

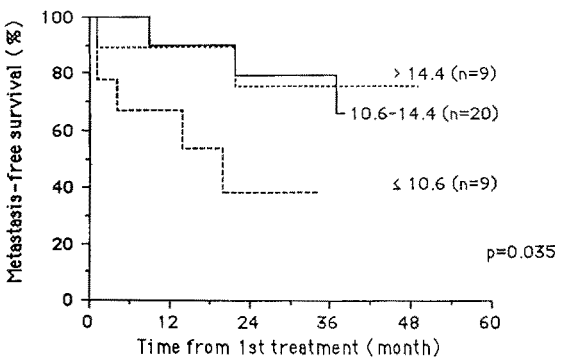

Fig. 4. Post-menopausal patients; probability of remaining metastasis-free according to stearic acid content of tumor phosphatidylcholine.

dex was deleted from the analysis, then histoprognostic grade became predictive in its place (p= 0.024). When the multivariate analysis was performed without entering stearic acid level, then elevated palmitic acid appeared as the only parameter independently linked with metastasis (p= 0.017).

Prognostic value of PC stearic acid level

The predictive value of the PC stearic acid level on metastasis occurrence and survival was therefore examined in a more detailed fashion. Since the two cut-off values were set at 14.4 and 10.6% of total fatty acids, the three populations of patients individualized were defined by a high (more than 14.4% of total fatty acids), moderate (between 10.6 to 14.4%), or low (less or equal to 10.6%) level of stearic acid in their tumor PC. When stearate level was low, the risk of developing metastasis was 5.2 times greater than when it was high, and the relative death rate was 5.9 times greater than when it was moderate (Table 2). The 3 year probability of remaining metastasis-free was 48, 70, and 84% when stearic acid level was low, moderate, or high (p= 0.033, Fig. 2a). Survival was also associated with stearic acid level, with 3year rates respectively of 50, 88, and 100% (p= 0.0004, Fig. 2b). The stearic acid content of PC was even more predictive of metastasis occurrence in patients with axillary lymph node invasion (Fig. 3), a homogeneous population by the stage of tumor extension (p= 0.003). When patients were stratified in two groups according to menopausal status, stearic acid level of PC remained predictive of metastasis occurrence only in post-menopausal patients (p= 0.035, Fig. 4), although a trend was present in premenopausal patients.

Discussion

Fatty acid composition of tumors has been investigated in numerous studies carried out on experimental animal systems after the recognition of the ability of fatty acids to modulate both tumor growth and several aspects of the immune response potentially involved in the control of cancer extension [25]. In contrast, fatty acid composition of human breast tumors has seldom been reported, and no attempt has been made to relate it to the clinical behavior of the disease. In a preliminary report, we provided data showing that poor evolution (i.e., early metastasis subsequent to treatment) was more likely to occur when the level of n-6 polyunsaturated fatty acids was low in membrane phosphatidylethanolamine (PE) of the primary tumor at the time of initial surgery [8]. PE was the first lipid class examined because of its natural enrichment in unsaturated fatty acids. We now show that: 1) fatty acid composition of another phospholipid, phosphatidylcholine, is related to prognosis; 2) tumors with low stearic acid- or with high palmitic acid-containing molecular species of PC are likely to give metastasis during evolution in univariate studies; 3) only

low stearic acid level remains associated with relapse or death when all fatty acids are taken into account in multiple regression analysis; and 4) the predictive value of low stearate in membrane PC on metastasis risk is higher in node-positive patients than in the whole population of patients. It also remains predictive of metastasis in the subgroup of post-menopausal patients.

There are possible limits in measuring tumor fatty acid composition as an intra-tumor marker of prognosis. Breast tumors are heterogeneous, and sometimes multiple, and the fatty acid composition of the examined tumor fragment may be different from that of a vicinal tumor fragment at a later stage of tumor progression [26], which is more likely to be responsible for subsequent metastasis. However, this limitation applies to any intra-tumor marker. Secondly, because of technical constraints, tumors of small size cannot be presently analyzed, explaining the small number of patients with tumors ≤ 25 mm in this study. Most of all, the measurement of fatty acids is a long procedure, and its standardization for routine laboratory practice is difficult. A simplified assay has to be developed before tumor fatty acid levels became useful in clinical practice.

The independent predictive value of a low stearic acid level of tumor membrane PC on breast cancer prognosis is a puzzling finding. Low level of stearic acid has already been reported in blood cells of patients with malignancies, along with an elevated level of oleic acid, leading to the proposal that lowered stearate was the consequence of increased delta-9 desaturation to oleic acid [27]. It would be interesting to know whether palmitic acid was high in such patients, and whether the change was greater in specific lipid classes, such as phosphatidylcholine. We did not find any prognostic value to the ratio stearate/oleate in phosphatidylcholine of breast tumors. The possibility that stearic acid might influence tumor development has also been studied. Parenteral administration of stearic acid was shown to delay mammary tumor development in a chemical carcinogenesis animal model [28]. However, composition of membrane lipids of the tumors was not provided, and it is not known whether alterations occurred, and whether they were similar to those we describe in our patients who did not relapse.

Although the influence of dietary fat on tumor fatty acid composition is not known in humans, it can be reasonably assumed that very different diet would lead to changes in tumor membrane fatty acid composition. The association observed in the present study between tumor fatty acid composition and post-therapeutic evolution of breast cancer, together with epidemiologic evidence linking dietary lipids to post-therapeutic evolution [29] or presentation [30] of breast cancer, suggests that dietary lipids could modulate breast cancer evolution or response to treatment through alteration of the fatty acid composition of the carcinoma. Whatever the mechanism involved, further investigation is warranted to better identify the nature of the association between structural lipids of the primary tumor and the behavior of a common and frequent malignant disease.

Acknowledgements

This work was supported in part by grants from the 'Ligue Nationale Française Contre le Cancer' (LNFCC, Comités National et Départemental du Cher). V. Chajes was the recipient of a LNFCC fellowship (Comité du Cher). The contribution of F. Fetissof to the interpretation of histopathologic findings is acknowledged.

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