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LAPTM4B gene copy number gain is associated with inferior response to anthracycline-based chemotherapy in hormone receptor negative breast carcinomas

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Abstract

Purpose To determine the associations between lysosomal-associated transmembrane protein 4b (*LAPTM4B)* gene copy number and response to different chemotherapy regimens in hormone receptor negative (HR-) primary breast carcinomas.
Patients and methods Two cohorts were analyzed: (1) 69 core biopsies from HR-breast carcinomas treated with neoadjuvant chemotherapy (anthracycline based in 72.5% of patients and non-anthracycline based in 27.5% of patients). (2) Tissue microarray (TMA) of 74 HR-breast carcinomas treated with adjuvant therapy (77.0% of the patients received anthracycline, 17.6% of the patients non-anthracycline-based therapy, and in 5.4% of the cases, no treatment data are available). Interphase FISH technique was applied on pretreatment core biopsies (cohort I) and on TMAs (cohort II) using custom-made dual-labelled FISH probes (LAPTM4B/CEN8q FISH probe Abnova Corp.).
Results In the neoadjuvant cohort in the anthracycline-treated group, we observed a significant difference ($p = 0.029$) of average *LAPTM4B* copy number between the non-responder and pathological complete responder groups (4.1 ± 1.1 vs. 2.6 ± 0.1). In the adjuvant setting, the anthracycline-treated group of metastatic breast carcinomas was characterized by higher *LAPTM4B* copy number comparing to the non-metastatic ones ($p = 0.046$). In contrast, in the non-anthracycline-treated group of patients, we did not find any *LAPTM4B* gene copy number differences between responder vs. non-responder groups or between metastatic vs. non-metastatic groups.
Conclusion Our results confirm the possible role of the *LAPTM4B* gene in anthracycline resistance in HR− breast cancer. Analyzing *LAPTM4B* copy number pattern may support future treatment decision.

Keywords Breast carcinomas · LAPTM4B · Anthracycline-based chemotherapy · FISH

Introduction

In the 1990s, chemotherapy regimens containing anthracyclines largely replaced the previous regimen of cyclophosphamide, methotrexate, and 5-fluorouracil (CMF) for the

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treatment of breast cancer. Subsequently, anthracyclines (doxorubicin and epirubicin) became the most widely used chemotherapeutic agents for breast cancer given as either adjuvant or neoadjuvant therapy [1, 2]. However, in the era of personalized therapy, clinical decision could be significantly optimized by a robust and reliable biomarker predicting response to this therapy.

Several potential response predictors for anthracyclines, such as increased topoisomerase-II-alpha levels, were proposed, but none of those has reached or passed the stage of large-scale independent clinical validation with the desired predictive potential [3, 4].

Using an integrative genomics approach, Li et al. [5] identified a functionally validated predictor of anthracycline treatment efficacy. Amplification of chromosomal region

8q22 was significantly associated with treatment resistance. Expression levels of one of the genes contained in that region, the lysosomal-associated transmembrane protein 4b (LAPTM4B), could be linked to altering the intracellular distribution of anthracycline in experimental circumstances. Anthracyclines likely enter the cells via simple diffusion [6], where they bind to proteasomes and by an ATP-dependent nuclear pore-mediated mechanism get transported into the nuclei [7]. Both doxorubicin and epirubicin are weak bases; they can accumulate in acidic intracellular compartments, such as the lysosomes. Several studies reported that resistant cancer cells are able to accumulate significantly more anthracyclines in cytoplasmic organelles, resulting in reduced nuclear drug accumulation and decreased cytotoxicity [8], but the potential molecular regulation of the drug sequestration in acid lysosomes has been unclear. In the previous experiments, the knockdown of LAPTM4B significantly decreased the ability of lysosomes to retain doxorubicin [5]. According to other results, the preservation of lysosome membrane integrity by LAPTM4B also prevents cathepsin release and the following caspase-mediated apoptosis, which was detectable after doxorubicin but not taxol exposure [9].

In the current study, we investigated whether we could potentially convert the initial observations of Li et al. [5] into a diagnostic test that is widely available in the diagnostic setting: FISH-based copy number analysis. To achieve this, first, we needed to determine whether the LAPTM4B/CEN8q ratio or the average copy number of LAPTM4B/cell was more robustly informative of treatment response. The ultimate aim of the study was to determine whether the presence of extra copy of LAPTM4B gene had negative predictive value to anthracycline-based treatment in patients with hormone receptor negative (HR−) breast carcinomas.

Patients and methods

Study population

A total of 143 HR− breast carcinoma cases were enrolled in this study and were analyzed in two different cohorts. The study was ethically approved by the Semmelweis University Institutional Review Board (SE-TUKEB 120/2013).

The first cohort included 69 core biopsies of HR− (64 TNBC and 5 HER2+) primary breast carcinoma cases diagnosed between 2004 and 2016, who received at least two cycles of neoadjuvant chemotherapy, and then underwent surgery. Patients were eligible for neoadjuvant therapy if they had histologically confirmed invasive breast cancer and imaging examinations ruled out distant metastases.

Fifty out of sixty-nine (72.5%) patients were treated with anthracycline-based neoadjuvant chemotherapy (mainly in combination with taxane), whereas 19 patients (27.5%) represented the control arm receiving non-anthracycline-based (mostly platinum in combination with taxane) chemotherapy. The clinicopathological data are presented in Table 1. The detailed treatment data are presented in supplementary Table 1.

The degree of response to therapy was categorized following Pinder et al. [10]. Complete pathological response (pCR) either (1) no residual carcinoma in breast and lymph nodes or (2) no residual invasive tumor, but DCIS present in breast and the absence of any residual invasive tumor in the lymph nodes. Partial response to therapy (pPR) either (1) minimal residual disease∕near total effect (e.g., < 10% of tumor remaining) or (2) evidence of response to therapy but with 10–50% of tumor remaining or (3) > 50% of tumor cellularity remains evident, when compared with the previous core biopsy sample, although some features of response to therapy present. No evidence of response to therapy (pNR).

The second cohort included 74 formalin-fixed paraffin-embedded (FFPE) samples of surgically removed HR− breast carcinomas (39 TNBC, 27 HER2+, and 8 with no reliable HER2 data). Patients in this cohort were treated

Table 1 Clinicopathological characteristics of the study group treated with neoadjuvant chemotherapy

Parameter	No. of patients ($N=69$)
Mean age (years)	50 (range: 26–79)
IHC-based molecular types	
TNBC	64 (92.8%)
HR−, HER2+	5 (7.2%)
Clinical tumor stage	
Primary tumor	
T1	2 (2.9%)
T2	42 (60.9%)
T3	12 (17.4%)
T4	13 (18.8%)
Regional lymph node	
N0	25 (36.2%)
N1	19 (27.5%)
N2	19 (27.5%)
N3	6 (8.8%)
Therapy	
Anthracycline-based	50 (72.5%)
Non-anthracycline-based	19 (27.5%)
Pathological response to neoadjuvant therapy	
pCR	26 (37.7%)
pPR	38 (55.1%)
pPRi	6 (15.78%)
pPRii	13 (34.21%)
pPRiii	19 (50%)
pNR	5 (7.2%)

with chemotherapy in adjuvant setting between 1999 and 2006. Of these patients, 57/74 (77.0%) received anthracycline-based (22.8% in combination with taxane) and 13/74 (17.6%) received non-anthracycline-based therapies (as control arm), mainly CMF regimens. In 4/74 (5.4%) cases, no reliable treatment data were available. The clinicopathological data are presented in Table 2. The detailed treatment data are presented in supplementary Table 2. In the adjuvant cohort, distant metastasis-free survival (DMFS) was assessed and defined as the time elapsed between the first diagnosis of primary breast carcinoma and the date of appearance of any distant metastasis. The occurrence or absence of distant metastasis was considered as an indirect surrogate marker for response to different chemotherapy regimens.

Follow-up data collection for both cohorts ended in December 2016.

Table 2 Clinicopathological characteristics of the study group treated with adjuvant chemotherapy

Parameter	No. of patients ($N = 74$)
Mean age (year)	52 (range: 32–81)
IHC-based molecular types	
TNBC	39 (52.7%)
HR−, HER2+	27 (36.5%)
HR−, HER2 n.a.	8 (10.8%)
Pathologic tumor stage	
Primary tumor	
pT1	19 (25.6%)
pT2	37 (50.0%)
pT3	9 (12.2%)
pT4	5 (6.8%)
Unknown	4 (5.4%)
Regional lymph node	
pN0	19 (25.6%)
pN1	21 (28.4%)
pN2	17 (23.0%)
pN3	4 (5.4%)
Unknown	13 (17.6%)
Distant metastases	
Yes	30 (40.5%)
No	44 (59.5%)
Therapy	
Anthracycline-based	57 (77.0%)
Non-anthracycline-based	13 (17.6%)
Unknown	4 (5.4%)

Evaluation of LAPTM4B copy number using FISH technique

Interphase FISH analysis was used to evaluate the copy number status of *LAPTM4B* gene.

5 μm-thick FFPE tissue sections were mounted onto Superfrost Plus positively charged slides, and deparaffinized and rehydrated in distilled water. For antigen retrieval, sections were incubated in citric acid-based antigen unmasking solution (Vector Laboratories, Inc. Burlingame, CA, USA) at 95 °C for 20 min. Cell lysis was established by incubating the sections in Triton X-100 (AppliChem GmbH, Ottoweg 4, 64291 Darmstadt, Germany) –SSC solution at 65 °C for 30 min. Sections were then subjected to digestion in pepsin solution, for 12 min at 37 °C, and then washed twice in SSC for 5 min. ZytoLight® FISH-Tissue Implementation Kit (ZytoVision GmbH, Bremerhaven, Germany) was used in prehybridizational steps. Sections were air-dried prior to denaturation at 73 °C for 10 min. Hybridization was performed using 4 μl of custom-made, Texas Red/FITC dual-labelled LAPTM4B/CEN8q FISH probes (Abnova Corp., Taoyuan City, Taiwan) per slide at 37 °C for 16–18 h in an automated hybridization chamber (ZYTOMED Systems GmbH Berlin, Germany). Slides were then immersed in wash buffer SSC for 30 min at 45 °C, rinsed in water for 10 min, air-dried. Cell nuclei were counterstained with DAPI in antifade solution (Vector Laboratories, Inc. Burlingame, CA, 94010, USA).

The Leica DM RXA fluorescent microscope equipped with Leica DFC 365FX high-performance CCD camera (Leica Microsystems GmbH, Wetzlar, Germany) and with DAPI long-pass, FITC, and Texas Red filters was used to evaluate the hybridization results.

Areas with well-separated cell nuclei and overall good hybridization signals were selected for analysis. Minimum two FISH images per case were digitally captured at 63x magnification. For each case, red (LAPTM4B) and green (CEN8 centromeric region) fluorescent signals were counted separately in at least 50 non-overlapping interphase nuclei. Based on these data, the following parameters were calculated: average *LAPTM4B* copy number/cell, average CEN8q copy number/cell, *LAPTM4B*/CEN8q ratio, average *LAPTM4B* copy number/cell in amplified cell population, and percentage of polysomic or amplified cells.

Statistical analysis

SPSS 23.0 (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. The Shapiro–Wilk test was applied to control the normality of data, and Leven's test was used to control the equality of variances. Non-parametric tests were applied for statistical analyses. Receiver-operating characteristic (ROC) curve analysis was used to determine the optimal

cut-off value of *LAPTM4B* copy number used in survival tests. Fisher's exact, Kruskal–Wallis, and Mann–Whitney–Wilcoxon tests were used to compare the *LAPTM4B* copy number to the clinical-pathological data of the primary tumors, including therapy response in neoadjuvant cohort and distant metastasis formation in adjuvant cohort as end point. The Kaplan–Meier, log-rank, and Cox regression tests were used for survival analysis (DMFS—distant metastasis-free survival). The Cox model included the known breast carcinoma prognostic factors such as age at the initial diagnosis, pT, and pN status beside *LAPTM4B* copy number category. A *p* value of less than 0.05 was considered statistically significant.

Results

In the first cohort, the mean age of patients was 50 years (range: 26–79 years); all cases had invasive carcinoma of no special type. Based on the subtypes, 64/69 (92.8%) TNBC and 5/69 HER2+ (7.2%) cases were analyzed. After neoadjuvant therapy, pCR was achieved in 26 cases (37.7%), pPR in 38 cases (55.1%), and pNR in 5 cases (7.2%) (Table 1 and Suppl. Table 1). The average *LAPTM4B*/CEN8q ratio was ≥ 2.0 in only 6/69 (8.6%) cases with the highest ratio being 3.71.

Considering the average *LAPTM4B* copy number/cell in the group of patients receiving anthracycline-based neoadjuvant therapy, higher average *LAPTM4B* gene copy number

was observed in the pNR group compared to pCR group (4.1 ± 1.1 vs. 2.6 ± 0.1, $p = 0.029$) (Figs. 1a, b, 2a).

We also compared average *LAPTM4B* gene copy numbers between patients who had no regression or who presented minimal response to anthracycline-based neoadjuvant therapy (> 50% residual tumor remaining) (pNR + pPRiii) versus cases with pCR. Again, significantly higher average gene copy number was found in the group of patients with inferior response to anthracycline-based neoadjuvant therapy (3.3 ± 0.3 vs. 2.6 ± 0.1, $p = 0.035$, Fig. 2c).

The same is true for average CEN8q being significantly higher in the pNR and pNR + pPRiii groups compared to pCR group (3.7 ± 0.9 vs. 2.2 ± 0.1, $p = 0.048$ and 2.9 ± 0.3 vs. 2.2 ± 0.1, $p = 0.040$ respectively).
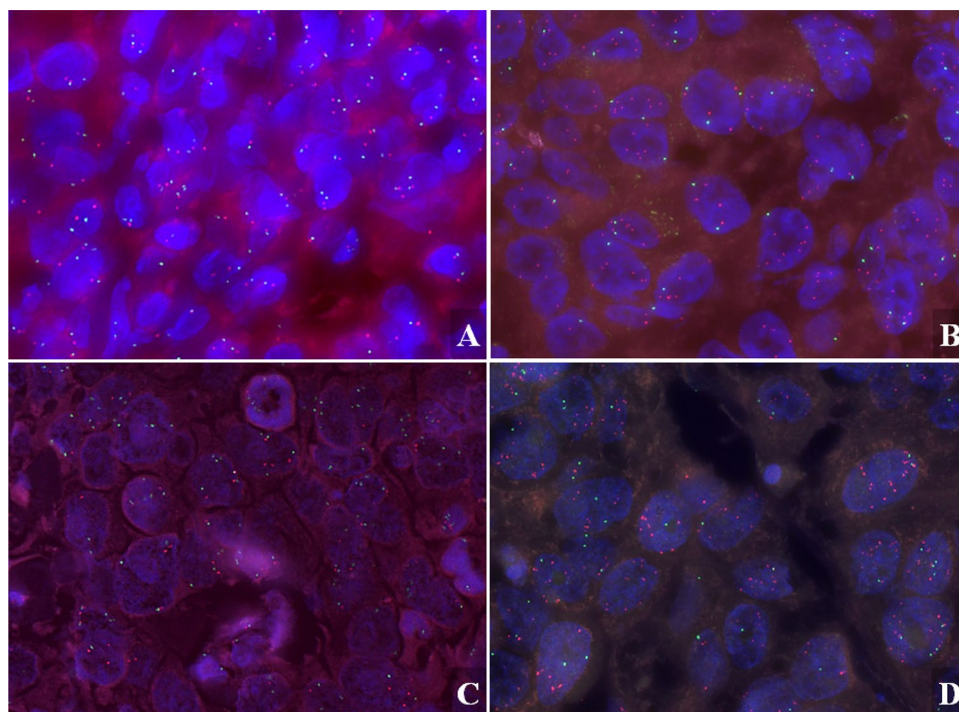
In the non-anthracycline-treated group of patients, we observed pNR in a single case (Fig. 2b). Therefore, we compared *LAPTM4B* gene copy number between pNR + pPRiii and pCR groups, resulting no significant differences ($p = 0.360$) (Fig. 2d).

Regarding average CEN8q copies, in the non-anthracycline-treated group of patients, no significant differences were observed between pNR + pPRiii and pCR groups ($p = 0.879$).

In the second cohort, the mean age of the patients was 52 years (32–81). Of the 74 analyzed cases, 94.6% had invasive carcinoma of no special type and 5.4% of the cases presented other histological type ($n = 1$ invasive lobular carcinoma, $n = 1$ carcinoma anaplasticum, $n = 1$ carcinoma medullare, $n = 1$ apocrin carcinoma). By considering the breast carcinoma subtypes, 39/74 (52.7%) were TNBC,

Fig. 1 *LAPTM4B* FISH images in anthracycline-treated cases. **a** Normal *LAPTM4B* copy number in a core biopsy case diagnosed with pCR after neoadjuvant therapy and **b** higher average *LAPTM4B* gene copy number in a case without any therapy response after neoadjuvant therapy; **c** a primary breast carcinoma case treated with adjuvant chemotherapy and presenting no distant organ metastasis during the follow-up period (**d**) and a primary breast carcinoma case who had higher average *LAPTM4B* gene copies and recurrence in distant organs. *LAPTM4B* gene was labelled with red, whereas chromosome 8 centromeric region was stained with green fluorescent dye. Cell nuclei were counterstained with DAPI (blue). FISH photos were acquired using 63× objective
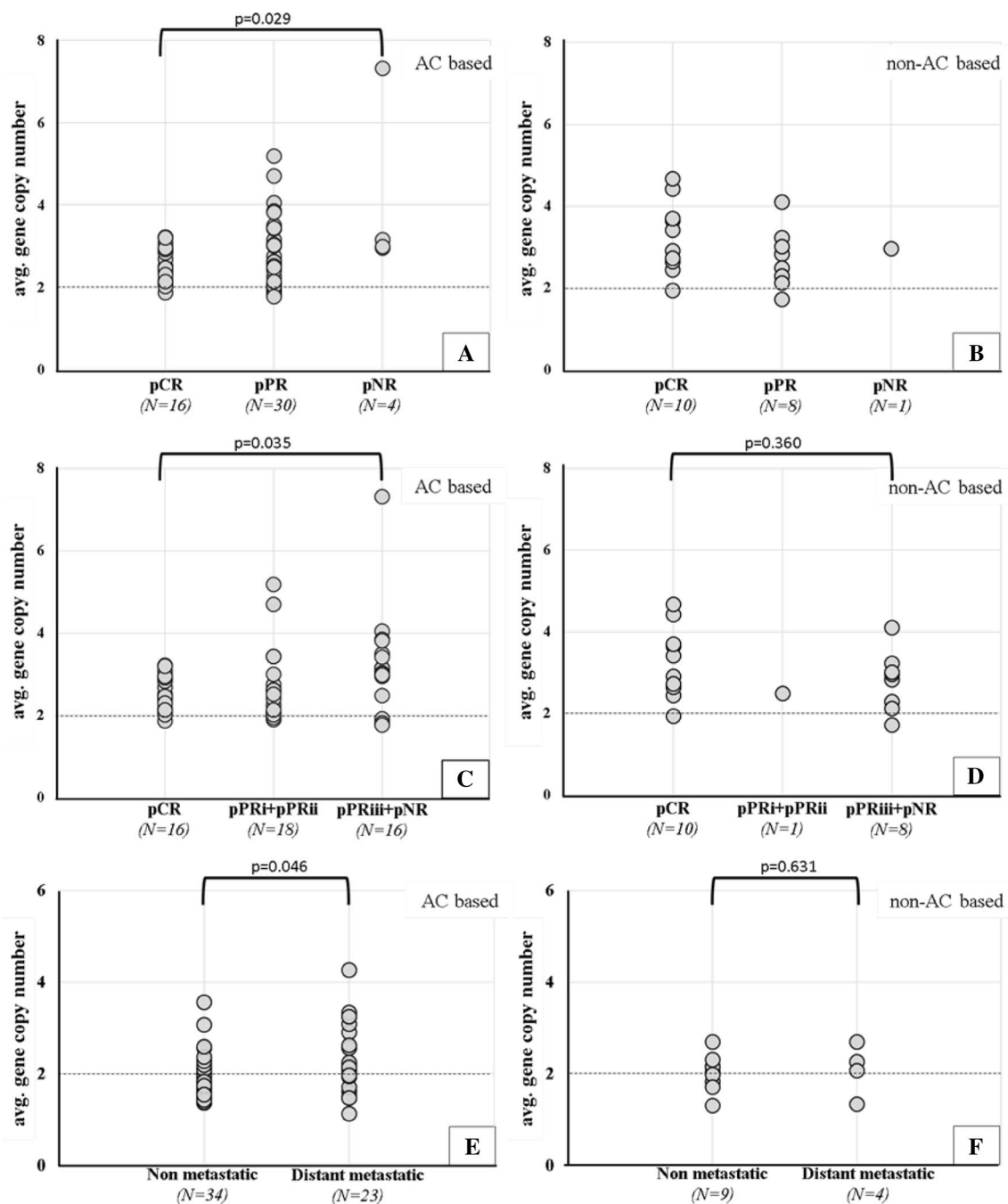
Fig. 2 Association between average *LAPTM4B* copy number and therapy response in the neoadjuvant (**a–d**) and in the adjuvant cohort (**e, f**). **a** In the anthracycline-treated cohort, significantly higher average *LAPTM4B* gene copy number was observed in pNR group compared to pCR group ($p = 0.029$). **b** In the non-anthracycline-treated group of patients, we observed pNR in a single case. **c** Significantly higher average *LAPTM4B* gene copy number observed in pNR + pPRiii group compared to pCR group ($p = 0.035$) in the anthracycline-treated cohort, whereas, in the non-anthracycline-treated cohort, (**d**) no significant differences were observed between the two groups. **e** Average gene copy number was significantly higher in metastatic cases, comparing to the non-metastatic ones in anthracycline-treated cohort ($p = 0.046$) and no significant differences in the non-anthracycline-treated cohort (**f**). A $p \leq 0.050$ was considered statistically significant using two-sided Mann–Whitney–Wilcoxon exact test

27/74 (36.5%) HER2 +, and in 8/74 (10.8%) cases, the HER2 status was unknown. During the follow-up period, distant metastases occurred in 30 (40.5%) cases (Table 2 and Suppl.

Table 2). *LAPTM4B*/CEN8q ratio ≥ 2.0 was observed in only 4/74 (5.4%) cases. Again considering the average *LAPTM4B* gene copy number in the adjuvant anthracycline-treated

patient cohort, the average *LAPTM4B* gene copy number was higher in metastatic cases, compared to the non-metastatic ones (2.2 ± 0.2 vs. 1.9 ± 0.1, $p = 0.046$, Figs. 1c, d, 2e). In patients treated with other than anthracycline chemotherapy, no significant differences were detected between metastatic vs. non-metastatic groups (Fig. 2f).

Regarding average CEN8q copies, no significant differences were observed between metastatic vs. non-metastatic groups neither in anthracycline-treated nor in non-anthracycline-treated patients.

Comparison of the two HR− subtypes (HER2+ and TNBC cases) showed no significant differences in the average *LAPTM4B* gene copy number/cell ($p = 0.328$).

Kaplan–Meier curve estimation based on DMFS revealed that higher *LAPTM4B* copy number was an independent predictor for DMFS in the anthracycline-treated adjuvant cohort (Log-rank test, $p = 0.037$). Cut-off value for poor prognosis was defined as follows: the ratio of amplified cell (*LAPTM4B*/CEN8q $\geq$ 2.0) population is more than 15% and the average gene copy number is more than 2.5 per sample (Fig. 3). Based on these criteria, of the 22/57 patients treated with anthracycline-based adjuvant chemotherapy and diagnosed with distant metastases, 6/22 cases presented higher *LAPTM4B* gene copy number, whereas, in 16/22 cases, lower *LAPTM4B* gene copy number was detected. Cox regression analysis was also performed, revealing association between increased *LAPTM4B* gene copy number and DMFS ($p = 0.044$).
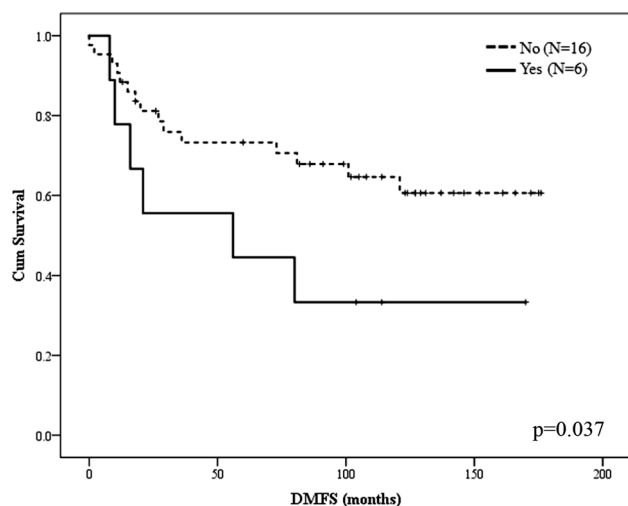

Fig. 3 Kaplan–Meier curve estimation of DMFS in the anthracycline (AC)-treated adjuvant cohort. Cut-off value for poor prognosis was defined as follows: the ratio of amplified cell (*LAPTM4B*/CEN8q $\geq$ 2.0) population is more than 15% and the average gene copy number is more than 2.5 per sample. Based on this criterion, 6/22 cases presented higher *LAPTM4B* gene copy number, whereas, in 16/22 cases, lower *LAPTM4B* gene copy number was detected (log-rank test, *p* value was 0.037)

Discussion

Anthracyclines are still the most widely used chemotherapeutic agents given as either adjuvant or neoadjuvant therapy [11, 12]. Considering that approximately 30% of the early stage breast cancers progress to the metastatic stage, and the fact that the response rate to anthracycline or taxane-based chemotherapy ranges from 30 to 70%, predictors of treatment efficacy to the various chemotherapeutic agents would have significant clinical importance [1, 3, 13, 14].

The potential role of LAPTM4B in tumor progression and chemotherapy resistance was first recognized about a decade ago. Kasper et al. [15] by analyzing different tumor types found that LAPTM4B was upregulated in 88% (23/26) of lung, in 67% (18/27) of colon carcinoma patients, and in the majority of endometrial (30/44), breast (27/53) and ovarian (11/16) carcinomas. They concluded that LAPTM4B have a dual functional involvement in tumor cell proliferation as well as in multidrug-resistance. Later, a significant functional association was found between lysosomal LAPTM4B overexpression and poor response to anthracycline chemotherapy in a neoadjuvant chemotherapy trial [5]. Although more and more new LAPTM4B mechanisms of action have been described, several unanswered questions have still remained.

One proposed mechanism of action by which LAPTM4B may confer anthracycline resistance is the cytosolic retention of the drug, thereby reducing the drug-induced DNA damage [16]. A different study proposed that the interaction of LAPTM4B-35 isoform with MDR1 could result in increased drug efflux. In addition, the activation of the PI3K/AKT signaling pathway by LAPTM4B-35 was proposed as anti-apoptotic mechanism [17, 18]. Li et al. have found that knockdown of *LAPTM4B* in MDA-MB-231 and BT549 cell lines has lead to elevated nuclear localization of doxorubicin [5]. Moreover, Li et al. found that LAPTM4B is required for lysosome homeostasis, acidification, and function. By limiting lysosome-mediated cell death and promoting autophagy, the protein has a significant effect on cancer cell survival, including greater resistance to nutrient deprivation, hypoxia, or chemotherapy-induced genotoxic stress [9].

Based on the results obtained from the analysis of our neoadjuvant cohort treated with anthracycline-based chemotherapy, and documenting higher average *LAPTM4B* copy number in the non-responder group and in pPR responders where > 50% of tumor cellularity remains evident, it seems reasonable to consider that LAPTM4B may contribute to chemoresistance against anthracyclines. In accordance with the results of Li et al. [5], we found that LAPTM4B alterations were not associated with treatment response

in the non-anthracycline-treated group of patients. However, the association of LAPTM4B overexpression with tumor progression is more speculative, and there is only a limited amount of supporting information in this regard [17, 19, 20]. In the present series of HR− breast carcinomas, we observed that, in the anthracycline-treated adjuvant cohort, the average gene copy number was higher in primary breast carcinomas where distant metastases were diagnosed during the follow-up period compared to the non-metastatic ones. It is questionable whether the association of *LAPTM4B* with poor prognosis is a consequence of anthracycline resistance or, as it is presented by Xiao M. et al., LAPTM4B has other mechanisms of action to promote cell proliferation, migration, invasion, leading to simultaneous upregulation of N-cadherin, vimentin, and downregulation of E-cadherin in T47D cells [20].

Li et al. found that elevated level of LAPTM4B and YWHAZ (another gene localized on 8q22) mRNAs was associated with shorter DMFS in women treated with adjuvant anthracycline chemotherapy. They also questioned whether this is a prognostic effect or a role of this two genes in chemotherapy resistance [5]. In other tumor types, such as hepatocellular- and ovarian carcinomas, the prognostic role of LAPTM4B was documented [21, 22]. Although Xiao M et al. showed that high LAPTM4B expression level is an independent predictor of axillary lymph node metastasis in breast carcinoma patients, unfortunately, no data were presented related to different breast cancer subtypes. In our study, only HR− breast carcinomas were analyzed and we found no significant difference of LAPTM4B copy number between HER2 + and TNBC subtypes. However, further analysis may be needed using higher number of cases.

The prognostic value of higher average CEN8q or chromosome 8 polysomy in breast carcinomas is not clear. We have found significantly higher average CEN8q copies in the group of patients with inferior response to anthracycline-based neoadjuvant therapy. Anna Batistatou et al. in 2017 have found that polysomy-8 was present in 39% HER2-positive tumors and in 30.2% HER2-negative tumors. They also showed that MYC gene amplification in the presence of chromosome-8 instability has distinct effects on patient outcome compared with MYC amplification with intact CEN8 [23].

In summary, the results of our study confirm the possible role of *LAPTM4B* gene in anthracycline resistance in HR− breast carcinomas. Alternative treatment modalities without anthracycline should be considered for those patients whose cancer harbors extra copies *LAPTM4B*.

Limitations of the study

Our results should be interpreted cautiously, since our patient cohort had relatively few cases in the pNR group.

Cases were retrospectively selected from prospectively maintained databases.

Compliance with ethical standards

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