Low level of Fibrillarin, a ribosome biogenesis factor, is a new independent marker of poor outcome in breast cancer

Flora Nguyen Van Long1,2,3, Audrey Lardy-Cleaud4, Dimitri Carène5,6, Caroline Rossoni5, Frédéric Catez1,2,3, Paul Rollet1,2,3, Nathalie Pion1,2,3, Déborah Monchier1,2,3, Agathe Dolbeau1,2,3, Marjorie Martin1,2,3, Valentin Simioni1,2,3, Susan Bray7, Doris Le Beherec5, Fernanda Mosele5, Ibrahim Bouakka5, Amélie Colombe-Vermorel9, Laetitia Odeyer9, Alexandra Diot10, Lee B. Jordan11, Alastair M. Thompson10,12, Françoise Jämen13,14, Thierry Dubois15, Sylvie Chabaud4, Stefan Michiels6, Isabelle Treilleux9, Jean-Christophe Bourdon10, David Pérot4, Alain Puisieux1,3, Fabrice André5, Jean-Jacques Diaz1,2,3* and Virginie Marcel1,2,3*

Abstract

Background: A current critical need remains in the identification of prognostic and predictive markers in early breast cancer. It appears that a distinctive trait of cancer cells is their addiction to hyperactivation of ribosome biogenesis. Thus, ribosome biogenesis might be an innovative source of biomarkers that remain to be evaluated.

Methods: Here, fibrillarin (FBL) was used as a surrogate marker of ribosome biogenesis due to its essential role in the early steps of ribosome biogenesis and its association with poor prognosis in breast cancer when overexpressed. Using 3,275 non-metastatic primary breast tumors, we analysed FBL mRNA expression levels and protein nucleolar organisation. Usage of TCGA dataset allowed transcriptomic comparison between the different FBL expression levels-related breast tumours.

Results: We unexpectedly discovered that in addition to breast tumours expressing high level of FBL, about 10% of the breast tumors express low level of FBL. A correlation between low FBL mRNA level and lack of FBL detection at protein level using immunohistochemistry was observed. Interestingly, multivariate analyses revealed that these low FBL tumors displayed poor outcome compared to current clinical gold standards. Transcriptomic data revealed that FBL expression is proportionally associated with distinct amount of ribosomes, low FBL level being associated with low amount of ribosomes. Moreover, the molecular programs supported by low and high FBL expressing tumors were distinct.

Conclusion: Altogether, we identified FBL as a powerful ribosome biogenesis-related independent marker of breast cancer outcome. Surprisingly we unveil a dual association of the ribosome biogenesis FBL factor with prognosis. These data suggest that hyper- but also hypo-activation of ribosome biogenesis are molecular traits of distinct tumors.

© The Author(s) 2022. Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if changes were made. The images or other third party material in this article are included in the article’s Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article’s Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit http://creativecommons.org/licenses/by/4.0/. The Creative Commons Public Domain Dedication waiver (http://creativecommons.org/publicdomain/zero/1.0/) applies to the data made available in this article, unless otherwise stated in a credit line to the data.
Background
Several studies have reported that increased protein synthesis induced by hyperactivation of ribosome biogenesis which occurs mainly within nucleoli, contributes to tumorigenesis by sustaining the hyperproliferative rate of cancer cells [1, 2]. The addiction of cancer cells to ribosome biogenesis hyperactivation is clearly illustrated by the numerous molecules developed in the last few years as cancer treatments that impair ribosome production either directly or indirectly [2–5]. Indeed, it has recently been shown that targeting ribosome biogenesis specifically kills cancer cells without affecting healthy ones [6, 7]. Moreover, a recent study revealed that oxaliplatin, conversely to other platinum-derived compounds, displays an anti-cancer activity revealed that oxaliplatin, conversely to other platinum-derived compounds, displays an anti-cancer activity. As a consequence, sensitivity of cancer cells to oxaliplatin is strongly correlated with expression levels of the different components making up the translation machinery [8]. Hyperactivation of ribosome biogenesis is a well-known marker of cancer cells [9]. Indeed, AgNOR staining (Argyrophillic Nucleolar Organiser region), which corresponds to silver staining of nucleolar regions where ribosome biogenesis takes place, is correlated with neoplastic transformation and cancer aggressiveness [9]. However, since such a correlation is not systematic, in particular in melanoma or mesothelioma [9], and automatic AgNOR staining and reading is still difficult to perform [10], this marker has never been approved for clinical purposes.

The ribosomal RNA (rRNA) methyltransferase fibrillarin (FBL) is one of the most abundant proteins present in nucleoli. This protein works as complex in concert with three proteins (NOP56, NOP58, NHP2L1) and exhibits several functional features, which are instrumental for ribosome biogenesis. On the one hand, FBL is one of the main regulators of several early steps of ribosome biogenesis, including ribosomal DNA (rDNA) synthesis and pre-rRNA cleavages [11–14]. On the other hand, FBL catalyzes the rRNA 2′-O-ribose methylation (2′-O-Me). It has also been reported that FBL expression is enhanced in prostatic neoplasia, in hepatocellular carcinoma and during mammary tumorigenesis [15–18]. In particular, tumors expressing high FBL levels are associated with poor outcome in breast cancer [15]. Moreover, we showed that FBL overexpression in MCF7 breast cancer cell lines promotes cell proliferation, colony formation and resistance to doxorubicin [15]. Indeed, we recently reported that alteration of FBL expression induces modulation of rRNA 2′-O-Me and directly affects translational activities of ribosomes thus altering the translation of specific mRNAs encoding oncogenic proteins such as IGF1R or CMYC [12, 15, 19]. FBL might thus represent a strong biomarker of ribosome biogenesis in cancer, in particular in breast cancers.

Although major advances have been made over the past 15 years, breast cancer still remains the most frequent cancer in women worldwide with about 2 million patients diagnosed in 2018 (Globocan 2018, OMS). Breast cancer-related death is intimately linked to the nature of the tumor since this heterogeneous disease encompasses several subtypes with distinct phenotypes, responses to therapy and thus clinical outcomes [20]. The strategy used for breast cancer patient management relies on the identification at diagnosis of breast cancer subtypes and characteristics to provide a therapeutic treatment specifically adapted to the tumor. Nevertheless 15–20% of breast cancer patients are still dying from their disease. One important issue for clinicians remains the identification of prognostic and predictive markers, including at early stage of the disease [21]. Here, we determine whether FBL is a marker of patient outcome at early stages of breast cancer that might reflect ribosome biogenesis activity.

Methods
Human breast tumors and healthy donors samples
A total of 3,275 primary breast tumors non-metastatic at diagnosis, encompassing six cohorts of breast cancer patients from four different institutions were analyzed: Tayside Tissue Bank of Dundee (TTBD, Dundee, Scotland, UK), The Cancer Genome Atlas (TCGA, NIH, USA) [22], BB-0033–00050 CRB Centre Léon Bérard (CLB, Lyon, France) and Institut Gustave Roussy (IGR, Villejuif, France) (Supplementary Table S1). A seventh series composed of 11 mammary tissues derived from healthy donors and issued from reduction mammoplasties was provided by the Institut Curie (Paris, France) [23]. Detailed information is available in the Supplementary Material and Methods.

Gene expression analysis
Gene expression was quantified by medium-throughput real-time quantitative PCR using the HD Biomark system (Fluidigm) as in [24]. Relative fold-changes were calculated using the 2-ΔΔCT method. Using RNA-seq data
derived from the TCGA series (RNA Seq V2 RSEM), transcriptomic analyses were performed using k-means clustering approach.

**FBL immunohistochemical staining**

FBL immunohistochemistry (IHC) was performed using two different lots of FBL antibody for CLB-1 (ab5821 lot GR1979-001, Abcam) and IGR-1 series (ab5821 lot GR253838-1, Abcam). FBL staining corresponds to a nucleolar staining. Breast tumors were classified according to FBL immunostaining organization, i.e., the number of FBL dots per nucleus. Four different types of FBL immunostaining categories were detected: "single" (1 dot per cell); "multiple" (> 1 dot per cell); "heterogeneous" (mix of "single" and "multiple"); and finally "no detection".

**Statistical analysis**

Descriptive statistics were used to summarize the initial characteristics of patients. Survival curves with associated log-rank tests were generated using the Kaplan Meier method for overall survival (OS: from diagnosis to death), invasive disease-free survival (iDFS: from diagnosis to either locoregional relapse, metastasis detection, new breast cancer or death), distant disease-free survival (dDFS: from diagnosis to occurrence of metastases or death) and disease-free survival (DFS: from diagnosis to either relapse or death from all causes if no relapse had been observed). Univariate and multivariate Cox proportional hazards models were used to investigate confounding factors predictive of survivals. Statistical analyses were performed using either SAS v9.4 (SAS Institute), R v3.5.1 (package survival) or GraphPad Prism v7.0a (GraphPad Software, Inc) software.

**Results**

**Lowest levels of FBL mRNA are associated with poor patient outcome at early stage of breast cancer**

To decipher whether ribosome biogenesis factors, and in particular the rRNA methyltransferase FBL, could be exploited as a novel biomarker to identify breast cancer patients with the poorest outcome at an early stage diagnosis, we first analyzed the association between FBL mRNA expression and overall survival (OS) or disease-free survival (DFS) in a series of 216 breast tumors (TTBD series, Supplementary Table S1). We validated that the TTBD series displayed characteristics of a classical breast cancer population (data not shown).

Using the quartile and tercile distribution of FBL mRNA levels as initial cut-off values, we unexpectedly observed that three groups of FBL-related breast cancer patients exhibited different OS (quartile: \( P = 0.0725 \), tercile: \( P = 0.0745 \), Supplementary figure S1A-C). To avoid inclusion of borderline “intermediate” FBL-expressing tumors in both “low” and “high” FBL groups, more stringent FBL cut-off values were then refined to produce “low” [0–20%] (> 0 and ≤ 20%), “intermediate” [20–80%] (> 20 and ≤ 80%) and “high” [80–100%] (> 80 and ≤ 100%) FBL mRNA-related groups. Based on this FBL-related stratification, Kaplan–Meier curves revealed that patients harboring breast tumors expressing different FBL mRNA levels displayed distinct OS and DFS (\( P = 0.0128 \) and 0.0053, respectively, Fig. 1A-B). Univariate Cox regression analyses support this observation (Table 1, FBL set 1). Moreover, these data show that “low” and “high” FBL marker were associated with a hazard ratio (HR) > 1 when using the “intermediate” marker as a reference (OS: HR high: 1.40, CI95%: [0.84–2.34], HR low: 2.01, CI95%: [1.25–3.23], \( P = 0.0150 \); DFS: HR high: 1.51, CI95%: [0.93–2.45], HR low: 2.06, CI95%: [1.30–3.27], \( P = 0.0065 \)), suggesting that patients bearing tumors with either “low” or “high” FBL mRNA levels displayed poor OS and DFS than the ones bearing tumours with “intermediate” FBL mRNA levels.

A similar observation between FBL mRNA expression levels and OS/DFS was made using a second set of FBL primers (OS: \( P = 0.0577 \); DFS: \( P = 0.0774 \); Supplementary figure S1D-E). In a second independent series of 661 breast samples, breast tumors expressing low FBL mRNA levels also displayed poor survivals (TCGA series, OS \( P = 0.0057 \), DFS \( P = 0.0037 \); Fig. 1C-D). Association between poor OS/DFS and high FBL mRNA levels was observed for the first 5 years. To reinforce these data, we performed complementary statistical analyses. Using FBL data derived from the two sets of primers in the TTBD series, univariate Cox regression analyses showed an association between FBL mRNA levels, in particular between “low” FBL, and poor OS (FBL set 2: HR high: 1.53, CI95%: [0.92–2.54], HR low: 1.69, CI95%: [1.04–2.75], \( P = 0.0615 \)) and DFS (FBL set 2: HR high: 1.52, CI95%: [0.94–2.46], HR low: 1.58, CI95%: [0.99–2.53], \( P = 0.0815 \)) (Table 1).

To further characterize the three tumor groups, and particularly to determine how the levels of FBL mRNA from tumor cells diverged from those of healthy cells, we compared FBL mRNA levels quantified in the TTBD series to those of 11 mastectomy samples from healthy donors (Supplementary figure S1F). Compared to healthy tissues, a significant decrease in FBL mRNA levels was observed in “low” FBL expressing tumors (\( P < 0.0001 \)), suggesting that these tumors exhibit FBL underexpression. A significant gradual increase in FBL mRNA levels was observed in the “intermediate” and “high” FBL expressing tumors (\( P < 0.0001 \)), suggesting that the “high” FBL expressing tumors displayed overexpressed FBL mRNA levels compared to normal tissues. Altogether, these data suggest that overexpression, but also and
mainly underexpression, of FBL is associated with poor patient prognosis at an early stage of breast cancer.

**FBL is an independent marker of poor patient outcome in breast cancer at the mRNA level**

To determine whether FBL is an independent marker of breast cancer outcome, we first performed univariate Cox regression analyses for NHP2L1, NOP56 and NOP58, the three factors associated with FBL in the rRNA 2'-O-Me maturation complex (C/D box snoRNP complex) (Table 1) [12]. An association between NHP2L1 or NOP56 mRNA levels and OS or DFS was observed (NHP2L1: OS \( P = 0.0763 \) and DFS \( P = 0.0538 \), respectively; NOP56: OS \( P = 0.1165 \) and DFS \( P = 0.0604 \)). However, a stronger and significant association was observed using a univariate Cox model between NOP58 mRNA levels and OS (\( P = 0.0031 \)) and DFS (\( P = 0.0078 \)). A strong correlation between FBL and NOP58 mRNA levels was observed in the TTBD series (\( r = 0.68, P < 0.0001 \), data not shown). Multivariate Cox regression model was performed to decipher whether both FBL and NOP58 had each an independent effect on OS and/or DFS even after adjustment against gold standard prognostic factors, including tumor size, lymph node invasion status and breast cancer subtype (Tables 2 and 3). These models revealed that FBL, but not NOP58, remained associated with OS (Low

---

**Fig. 1** Association between FBL mRNA levels and survivals in two independent breast cancer series. Using the cut-offs identified in the Supplementary figure S2 for FBL mRNA expression levels, association between FBL mRNA levels and OS (A, C) and DFS (B, D) was determined using Kaplan–Meier analyses in the TTBD series (\( n = 216, A \ B \)) and TCGA series (\( n = 661, C \ D \)). An association between FBL mRNA expression and OS and DFS was observed in the two independent breast cancer series. Patients bearing breast tumors expressing either "low" or "high" FBL mRNA levels exhibited worse survival compared to tumors expressing "int" FBL mRNA levels. Int.: Intermediate. *: \( P < 0.05 \); **: \( P < 0.01 \).
**Table 1**  Association between rRNA 2'-O-Me maturation complex factors and overall survival or disease-free survival using univariate Cox regression analyses in the TTBD series

| Variables | Overall survival | Disease-free survival |
|-----------|-----------------|-----------------------|
|           | HR   | CI95%     | P-value | HR   | CI95%     | P-value |
| **FBL (set 1)** |       |           |         |       |           |         |
| Intermediate  | 1.00 |           | 0.0150* | 1.00 |           | 0.0065** |
| Low          | 2.01 | [1.25–3.23] |         | 2.06 | [1.30–3.27] |         |
| High         | 1.40 | [0.84–2.34] |         | 1.51 | [0.93–2.45] |         |
| **FBL (set 2)** |       |           |         |       |           |         |
| Intermediate  | 1.00 |           | 0.0615  | 1.00 |           | 0.0815  |
| Low          | 1.69 | [1.04–2.75] |         | 1.58 | [0.99–2.53] |         |
| High         | 1.53 | [0.92–2.54] |         | 1.52 | [0.94–2.46] |         |
| **NHP2L1**   |       |           |         |       |           |         |
| Intermediate  | 1.00 |           | 0.0763  | 1.00 |           | 0.0538  |
| Low          | 1.67 | [1.05–2.66] |         | 1.74 | [1.11–2.72] |         |
| High         | 1.05 | [0.63–1.75] |         | 1.20 | [0.74–1.93] |         |
| **NOP56**    |       |           |         |       |           |         |
| Intermediate  | 1.00 |           |         | 1.00 |           | 0.0604  |
| Low          | 1.52 | [0.93–2.50] | 0.1165  | 1.46 | [0.90–2.36] |         |
| High         | 1.54 | [0.96–2.46] |         | 1.67 | [1.07–2.61] |         |
| **NOP58**    |       |           |         |       |           |         |
| High         | 1.00 |           | 0.0031**| 1.00 |           | 0.0078**|
| Low          | 2.01 | [1.27–3.20] |         | 1.84 | [1.17–2.88] |         |

_HR Hazard Ratio, CI95% 95% of Confidence Interval_

**Table 2**  Multivariate Cox regression analyses for overall survival and disease-free survival using significant univariate variables in the TTBD series (Step with NOP58 that was removed from the model)

| Variables | Overall survival | Disease-free survival |
|-----------|-----------------|-----------------------|
|           | HR   | CI95%     | P-value | HR   | CI95%     | P-value |
| **FBL**   |       |           |         |       |           |         |
| Intermediate  | 1.00 |           | 0.0230* | 1.00 |           | 0.0149* |
| Low          | 2.16 | [1.24–3.74] |         | 2.02 | [1.21–3.39] |         |
| High         | 1.32 | [0.74–2.34] |         | 1.56 | [0.94–2.56] |         |
| **NOP58**  |       |           |         |       |           |         |
| Low          | 1.00 |           | 0.4034  | 1.00 |           | 0.3036  |
| High         | 1.27 | [0.73–2.22] |         | 1.32 | [0.78–2.24] |         |
| **Tumor size** |       |           |         |       |           |         |
| < 30 mm      | 1.00 |           | <0.0001***| 1.00 |           | 0.0004**|
| ≥ 30 mm      | 2.70 | [1.71–4.23] |         | 2.07 | [1.38–3.09] |         |
| **Lymph node invasion status** |       |           |         |       |           |         |
| N=0          | 1.00 |           | 0.0386* | 1.00 |           | 0.0180* |
| N≥1          | 1.63 | [1.02–2.60] |         | 1.64 | [1.09–2.47] |         |
| **Breast cancer subtypes** |       |           |         |       |           |         |
| ER+ PR±HER2- | 1.00 |           | 0.0616  | N/A  |           | N/A     |
| ER±PR±HER2+  | 1.05 | [0.62–1.76] |         | 1.88 | [1.08–3.26] |         |
| ER- PR- HER2- | 1.88 |           |         | 1.88 | [1.08–3.26] |         |

_HR Hazard Ratio, CI95% 95% of Confidence Interval_
FBL: HR: 2.35, CI95%: [1.41–3.92]; High FBL: HR: 1.27, CI95%: [0.72–2.24]; P = 0.0042) and DFS (Low FBL: HR: 2.02, CI95%: [1.21–3.39]; High FBL: HR: 1.56, CI95%: [0.94–2.56]; P = 0.0042* and 0.0149* respectively) Therefore, FBL was identified as the only gene among the rRNA 2’-O-Me maturation complex of prognostic value in breast cancer.

An independent association between FBL and patient survivals after adjustment against gold standard prognostic factors was also obtained using the second set of FBL primers in the TTBD series for OS (Low FBL: HR: 2.10, CI95%: [1.27–3.47]; High FBL: HR: 1.69, CI95%: [1.02–2.80]; P = 0.00215) (data not shown). Interestingly, FBL mRNA levels significantly discriminated patients with different outcomes exhibiting either small tumor size or no invaded lymph node at early stage diagnosis (P < 0.0001 and 0.0073, respectively) (Supplementary figure S2A-D). Indeed, the combination of FBL mRNA level and tumor size highlighted three categories of breast cancer patients: patients with the best OS and DFS that carry small tumors expressing intermediate FBL mRNA levels; patients with the poorest OS and DFS that carry large tumors expressing low FBL mRNA levels; and patients with intermediate survival exhibiting other combinations. We next observed that “low” and “high” FBL-related breast cancer groups seemed to exhibit the same pattern of OS and DFS in specific breast cancer subtypes, including in ER+PR±HER2+ (corresponding to luminal subtype, P = 0.2485 and 0.2358, respectively) and in ER- PR- HER2- (corresponding to triple negative subtype, P = 0.0441 and 0.0323, respectively) (data not shown). This observation was not noticed in ER±PR±HER2+ tumors (corresponding to HER2-amplified subtype, P = 0.3868 and 0.1370, respectively). Overall, these data indicate that the FBL mRNA level is an independent marker of poor patient outcome at an early stage of breast cancer.

A validation series composed of 198 primary breast tumors was analyzed to sustain these observations (IGR-2 series, Supplementary Table S1). Since few events occurred in the IGR-2 series, we focused on the association of FBL mRNA levels with distant disease-free survival (dDFS) and we used the tercile as cut-off values, which were different and less stringent than the ones used for the TTBD and TCGA cohorts (Supplementary figure S2E). Kaplan–Meier curves (P = 0.032) and univariate Cox regression analyses (Low FBL: HR: 4.04, CI95%: [1.32–12.40], P = 0.0150; High FBL: HR: 3.14, CI95%: [0.98–10.02], P = 0.0540) showed that patients carrying tumors expressing either “low” or “high” FBL mRNA levels exhibited a significantly poorer dDFS than patients with tumors expressing “intermediate” FBL mRNA levels. In addition, multivariate Cox regression models built on current clinical markers revealed that the

| Variables | Overall survival | Disease-free survival |
|-----------|-----------------|----------------------|
|           | HR   | CI95%     | P-value | HR   | CI95%     | P-value |
| FBL       |      |           |         |      |           |         |
| Intermediate | 1.00 |           | 0.0042* | 1.00 |           | 0.0149* |
| Low       | 2.35 | [1.41–3.92] |        | 2.02 | [1.21–3.39] |        |
| High      | 1.27 | [0.72–2.24] |        | 1.56 | [0.94–2.56] |        |
| NOP58     |      |           |         |      |           |         |
| Low       |      | removed   |         |      | removed   |         |
| High      |      |           |         |      |           |         |
| Tumor size |      |           |         |      |           |         |
| < 30 mm   | 1.00 |           |          | 1.00 |           |         |
| ≥ 30 mm   | 2.77 | [1.77–4.33] |        | 2.14 | [1.44–3.18] |        |
| Lymph node invasion status |      |           |         |      |           |         |
| N=0       | 1.00 |           | 0.0443* | 1.00 |           | 0.0174* |
| N≥1       | 1.61 | [1.01–2.55] |        | 1.64 | [1.09–2.48] |        |
| Breast cancer subtypes |      |           |         |      |           |         |
| ER+PR±HER2- | 1.00 |           | 0.0463* | N/A |           | N/A     |
| ER±PR±HER2+ | 1.06 | [0.63–1.79] |        | N/A |           | N/A     |
| ER-PR-HER2- | 1.94 | [1.12–3.35] |        | N/A |           | N/A     |

HR Hazard Ratio, CI95% 95% of Confidence Interval, N/A Not include in the model, removed NOP58 was included in the model but removed in step (a) of a backward step selection.
FBL mRNA levels remained a significant marker of dDFS (Low FBL: HR: 3.89, CI95%: [1.07–14.11], P=0.0390; High FBL: HR: 3.92, CI95%: [1.11–13.91], P=0.0344) (Supplementary Table S2). Altogether these data identify FBL as an independent marker of patient outcome at an early stage of breast cancer.

Lack of FBL protein detection is associated with poor patient outcome in breast cancer

FBL expression at the protein level was analyzed by IHC in two TMAs: a test series of 389 primary breast tumors (CLB-1) and a validation series of 1,759 tumors (IGR-1) displaying characteristics of a classical breast cancer population (Supplementary Table S3). Western blotting analysis highlighted the strong specificity and efficacy of FBL antibody for FBL protein detection (Supplementary figure S3). Using IHC, FBL staining allowed a clear evaluation of the FBL intracellular distribution that corresponds to nucleolus location as expected [10]. However, due to the high and diverse number of nucleoli per tumor cells, evaluating difference in FBL expression level using IHC remains sensitive.

Therefore, breast tumors were classified only on the basis of the different intracellular distributions of FBL in the tumor cells (Fig. 2A). FBL nucleolar staining exhibited either a single dot per cell (termed “single”), or multiple dots per cell (“multiple”) or a combination of single and multiple dots per cell (“heterogeneous”). We also identified samples in which the FBL signal was not detected (“no detection”). This latter group corresponded to 8.6% of tumor samples. A similar distribution of this “no detection” group was observed in the IGR-1 series, representing 12% of tumor samples (Supplementary figure S4A). These data supported the existence of breast tumors with undetectable FBL protein.

The association between the four breast cancer patient groups exhibiting different FBL staining and OS/DFS was analyzed using Kaplan–Meier curves (Supplementary figure S4B-C). No significant association was observed (P=0.0988 and 0.4796, respectively). However, the patients bearing tumors with “no detection” displayed the poorest survival. We thus compared survival of patients with tumors exhibiting FBL staining or not (Fig. 2B-F). In the CLB-1 series, patients with a “no detection” tumor status tended to have a poorer OS and DFS than those of patients carrying tumors in which FBL staining was detectable (P=0.0699 and 0.1721, respectively) (Fig. 2B-C). In the IGR-1 series, a significant association was observed using Kaplan–Meier and univariate Cox regression analyses between patients harboring “no detection” tumors and poor iDFS and dDFS (P=0.0333 and 0.0184, respectively) (Fig. 2D-F and Table 4). These data suggest that “no detection” is a marker of poor patient outcome at early stages of breast cancer patients.

To ensure that the “no detection” status did not arise from lack of nucleoli preservation during sample processing, we analyzed nucleoli organization in the CLB-1 series using Hematoxylin/Phloxine Saffon (HPS) staining that allows visualization of subcellular compartments including nucleoli. We observed that all tumor samples displayed nucleoli, including the ones for which no detection of FBL staining was observed, suggesting that lack of detectable FBL was not related to their absence (Supplementary figure S4D). Using total RNA extracted from 41 randomly chosen samples issued from the CLB-1 series, we determined that tumors in which the FBL signal was not detected by IHC, expressed significantly lower FBL mRNA levels than the ones in which FBL staining was detected (P=0.0063, Supplementary figure S4E). Overall, these data support the existence of primary breast cancer tumors lacking detection of FBL protein associated with low mRNA levels of FBL that are associated with poor patient outcome at early stages of breast cancer.

Breast tumors expressing the highest and the lowest FBL mRNA levels exhibit distinct clinical and biological characteristics

To characterize the primary breast tumors expressing the highest and the lowest FBL mRNA levels, we first compared clinical characteristics of breast patients in the TTBD, CLB-1 and IGR-1 series. In the TTBD series, no significant difference was observed in the different FBL mRNA-related tumor groups (data not shown). Conversely, in the CLB-1 and IGR-1 series, tumors classified as “no detection” (i.e., low FBL) were associated with aggressive cancers exhibiting larger tumors, older patients (CLB-1 series, P=0.033 and 0.012, respectively) and enrichment in triple negative breast tumors compared to tumors displaying FBL staining (IGR-1 series, P<0.0001). These data suggest that breast tumors expressing the highest and the lowest levels of FBL exhibit different biological and clinical characteristics.
Fig. 2 (See legend on previous page.)
Next, we analyzed the association between FBL mRNA-related groups and genomic or transcriptomic specificities of the tumors using TCGA series. At the genomic level, we first observed that breast tumors expressing low FBL mRNA levels had significantly lower number of copy alterations or mutation counts than breast tumors exhibiting high FBL mRNA levels, supporting that the two FBL-related tumor groups are different (Supplementary figure S5A-B). Using the transcriptome dataset of TCGA series and clustering approaches to compare gene expression profiles within the FBL mRNA-related groups of breast tumors, we observed that 4 out of the 10 gene-based clusters displayed different expression profiles between high and low FBL-related tumors (Fig. 3). These data indicate that the two FBL-related tumor groups have different biological properties. Interestingly the cluster 0 is enriched in genes encoding ribosomal proteins and proteins involved in translation as shown by gene ontology analyses. Comparison of median expression of the genes coding for the 80 human ribosomal proteins in the three FBL-related tumors showed a significant dose-dependent correlation between FBL mRNA expression levels (i.e., low, intermediate and high) and ribosomal protein expression levels, as expected due to the role of FBL in ribosome biogenesis [12] (Supplementary figure S5C). Overall, it appears that breast tumors overexpressing and underexpressing FBL exhibit different clinical characteristics and gene expression profiles, in particular regarding ribosome production and mRNA translation.

Discussion

Our data identify the rRNA methyltransferase FBL as a strong ribosome biogenesis-related prognosis biomarker in non-metastatic breast cancer patients. A significant association between FBL expression and prognosis was obtained from the analyses of 6 independent breast cancer series representing a total of 3,275 samples. Despite differences between the test and validation series (three different European cancer centres with usage of different quantifiable events, statistical approaches, biological materials and mRNA quantification techniques), the data enabled us to draw similar conclusions, thus strengthening our findings. Interestingly, FBL remains an independent marker of poor prognosis at early stages of breast cancer even after adjustment against routinely used clinical gold standards. Such observations suggest that FBL could provide additional information compared to taking only clinical gold standards into account and could thus drastically improve patient stratification. For instance, in combination with tumor size, FBL expression led to the identification of patients with the poorest outcome although they harbored small tumors generally associated with a low risk factor in breast cancer patients. These breast cancer patients might either benefit from treatment usually used for large tumors or display poor outcome due to side-effects of their current treatment. Indeed, some patients who are at a low-risk of recurrence derive only a small benefit from adjuvant chemotherapies, which may be outweighed by long-term toxicities [21]. Identification of innovative biomarkers in such populations, like FBL, would allow the delivery of optimal treatments and de-escalation of therapies.

One of the most intriguing results of this study remains the identification of aggressive tumors expressing low levels of FBL, representing about 10% of all breast tumors. Although the definition of normal tissues regarding breast cancer disease is still a matter of controversy [25], we suggest that low FBL expressing tumors display reduced FBL expression levels compared to normal tissues. The existence of breast tumors underexpressing FBL identified at mRNA levels was supported by FBL immunostaining in TMAs: i) similar proportion of breast tumors exhibiting no FBL signal in two different breast tumour series; ii) preservation of nucleoli during sample processing; and iii) significant reduction of FBL mRNA levels in these tumors compared to FBL-stained tumors.

So far, all studies, including ours, demonstrated that FBL is overexpressed in breast tumors and is associated with poor patient outcome [15, 17, 18]. These data were accumulated taking into account molecular mechanisms available at the time of their publication. Indeed, we showed that FBL overexpression altered rRNA 2’-O-Me profiles thus affecting intrinsic activity of ribosomes and translational efficacy of some oncogenic mRNAs such as IGF1R or CMYC [15]. Moreover, FBL overexpressing breast cancer cell lines exhibited increased cell proliferation and resistance to chemotherapy, reinforcing the

### Table 4

| Variables                        | Survival     | HR   | CI95%     | P-value |
|----------------------------------|--------------|------|-----------|---------|
| Overall survival (OS)            |              |      |           |         |
| no detection                     | 1.00         | 0.160| [0.40–1.17] |         |
| detection                        | 0.68         |      | [0.40–1.17] |         |
| Invasive Disease-free survival (iDFS) |              |      |           |         |
| no detection                     | 1.00         | 0.034*| [0.47–0.97] |         |
| detection                        | 0.67         |      | [0.47–0.97] |         |
| Distant Disease-free survival (dDFS) |              |      |           |         |
| no detection                     | 1.00         | 0.020*| [0.41–0.93] |         |
| detection                        | 0.62         |      | [0.41–0.93] |         |

HR Hazard Ratio, CI95% 95% of Confidence Interval
association between FBL overexpression and poor survivals [15]. Finally, the fact that FBL is an essential gene, the homozygous depletion of which is lethal [26–28], has so far prevented to formulate hypotheses regarding the putative role of FBL reduction in tumorigenesis. However, by performing a non-hypothesis-driven study using large sample sets, we identified unexpected reduction of FBL found in about 10% of all breast tumors. Interestingly, underexpressing FBL tumors are characterized by a reduction in ribosomal proteins compared to tumors overexpressing FBL. Thus, FBL might directly reflect amount of ribosomes in tumors, as expected due to the pivotal role of FBL in ribosome biogenesis [12]. We have also recently demonstrated that reduced expression of nucleolin (NCL), a regulator of RNA polymerase I activity finely regulating ribosome production, is associated with poor prognosis in breast cancer, supporting a relationship between reduced ribosome biogenesis and cancer outcome [24]. It has to be noted that in anal squamous cell carcinoma, molecular classification based on proteomic profile distinguish two groups displaying either low or high amount of ribosome and translation related proteins [29]. These data support the notion that low amount of ribosomes might be a new feature of some particular tumors.

Two non-mutually exclusive hypotheses could be proposed regarding the contribution of FBL reduction in tumorigenesis. First, association of low FBL with poor prognosis might result from a decrease in ribosome biogenesis. Several hereditary diseases are indeed characterized by reduction of the number of ribosomes associated with increased cancer susceptibilities although the cellular and molecular mechanisms by which quantitative alteration of ribosomes contributes to neoplastic transformation remain a matter of debate [9, 30]. Decreased in ribosomal content might either induces p53 activation that should be bypass for cell survival thus resulting in the selective loss of p53 and
acquisition of neoplastic transformation, or impairs translation of some specific mRNAs encoding oncogenes and tumor suppressors. Second, association of low FBL with poor prognosis might result from qualitative alterations of ribosomes. Indeed, we recently reported that reduction of FBL expression in HeLa cells alters rRNA 2’-O-Me profiles and thus translational regulation by ribosomes [19]. Although rRNA 2’-O-Me profiles have not been compared in the same cell lines in response to alterations of FBL expression, it appears that sites exhibiting variations in rRNA 2’-O-Me level in response to a reduction or increase in FBL expression were different [15, 19]. Thus, overexpression and underexpression of FBL might differentially affect ribosome translational activities, including the rate of translation speed, by differentially modulating rRNA 2’-O-Me profiles. Hence, both alterations can support the high proliferative rate of cancer cells and promote development of tumors with distinct characteristics. Our data demonstrating that FBL, but not the other components of the rRNA 2’-Me maturation complex, is an independent marker of poor prognosis, support the important role of FBL in cancer. Such hypotheses regarding its related biological functions remain to be tested in the near future to sustain a dual role for FBL in tumorigenesis.

Conclusions

FBL appears as a novel independent marker of poor patient outcome in breast cancer that belongs to the emerging field of ribosome in oncology. In contrast to AgNOR reflecting nucleoli sub-cellular compartments that are always present in cancer cells but with different shapes and numbers rendering histological reading difficult, FBL expression corresponds to a classical gene-based biomarker easily applicable in the clinic. Furthermore, based on the recent demonstration that targeting ribosome biogenesis primarily through DNA integrating molecules is a specific and efficient strategy to target cancer [3, 4], our discovery may pave the way for therapeutic opportunities by directly targeting FBL, in particular in high FBL expressing breast cancers. Finally, the identification of breast tumors expressing low levels of FBL suggests that reduced amount of ribosomes might be a novel molecular feature of a particular set of tumors.

Abbreviations

AgNOR: Argyrophilic nucleolar organiser region; DFS: Disease-free survival; dDFS: Distant disease-free survival; ER: Estrogen receptor; FBL: Fibrillarin; HR: Hazard ratio; CI95%: Confidence interval 95%; iDFS: Invasive disease-free survival; OS: Overall survival; PR: Progesterone receptor; rRNA: Ribosomal RNA.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12885-022-09552-x.

Additional file 1: Supplementary Methods. Supplementary figure 1. Association between FBL mRNA levels and patient survival. Supplementary figure 2. Improvement of breast cancer patient stratification derived from tumor size and lymph node invasion status using FBL mRNA expression. Supplementary figure 3. Validation of FBL antibodies used for FBL immunostaining. Supplementary figure 4. Association between FBL immunostaining and patient survival in CLB-1 and IGR-1 series. Supplementary figure 5. Characterization of the three tumor groups expressing different FBL mRNA levels. Supplementary table 1. Characteristics of patients from TTBD, IGR-2 and TCGA RNA breast cancer series. Supplementary table 2. Multivariate Cox regression analyses of FBL mRNA expression and gold standard prognostic factors with distant disease-free survival in IGR-2 series. Supplementary table 3. Characteristics of patients from the CLB-1 and IGR-1 breast cancer series.

Acknowledgements

We thank platforms’ staffs that have not been referred as co-authors: Tayside Tissue Bank (TTBD), Dundee, Scotland, UK), CRB Centre Léon Bérard (Lyon, France, Dir: Dr S. Tabone-Eglenger), CRCL Microscopy facilities (CRCL, Lyon, France, co-Dir: Dr C. Vanbelle); BioCOS Life Sciences (Bioinformatics, Bengaluru, India); English editing (CRCL, Lyon, France, B. Manship). We thank Emilie Lavergne for her help in biostatistics at the beginning of the project.

Authors’ contributions

FNVL, FC, AD, MM, PR, NP, DM, VS, ACV, LO and JT performed and analysed experiments. FNVL, ALC, DC, CR, SC and DP performed statistical analyses. SB, DLB, FM, IB, AD, LBJ, AMT, FI, TD, IT, JCB, AP and FA provide human samples. FNVL, IT, FA, JJD and VM analysed and interpreted the data. JJD and VM shaped the research question and coordinated the project. FNVL, IT, SC, SM and VM supervised the daily experiments. FNVL, JJD and VM wrote the first draft of the manuscript. All authors have read and approved the manuscript.

Funding

The access to human series, clinical data, statistical analyses and reagents for experiments were funded by PAR Sein program (RiboTEM: ARC_INCa_ LNCC_7625), PLBio program (MARACAS: INCA PLBIO19-178), PRT-K program (EMT-ConCEPT: INCa PRT K17 2018-024), SIRIC program (INCA-DGOS-Inserm_12563, LyRCAN), Fondation ARC (PIA 20161204686) and Ligue Contre le Cancer Comité Isère then Fondation pour la Recherche Médicale.

Availability of data and materials

TCGA series corresponded to 818 samples obtained from TCGA Breast Invasive Carcinoma project of the public database cBioPortal (https://cbioportal.org).

Declarations

Ethical approval and consent to participate

All the experiment protocols for involving human data was in accordance with the guidelines of national and institutional entities based on Declaration of Helsinki in the manuscript. All experimental protocols were approved by the Ethics Committee of Ninewells Hospital, the French National Commission on Informatics and Liberty, the French National Institute of Cancer Ethic Charter and the ethics committee of the Institut Curie and IGR. Written informed consent was obtained from all patients at the diagnosis to the inclusion of their biological material.

Consent for publication

Not applicable.

Competing interests

The authors declare no potential conflict of interest.
References

1. Barria M, Pusic A, Zollo O, Costa M, Kondrashov N, Rego E, et al. Suppression of Myc oncogenic activity by ribosomal protein hpolinsufficiency. Nature. 2008;456:971–5. https://doi.org/10.1038/nature07449.

2. Truitt ML, Ruggero D. New frontiers in translational control of the cancer genome. Nat Rev Cancer. 2018;18:51–63. https://doi.org/10.1038/s41561-017-01027-z.

3. Aran D, Camarda R, Odegaard J, Paik H, Oskotsky B, Krings G, et al. Genetic profile and functional proteomics of anal squamous cell carcinoma. Oncol Lett. 2021;21(2):92. https://doi.org/10.3892/ol.2020.12353.

4. Friboulet F, Noyot Y, Hugel E, Drakos S. Ribosome biogenesis key molecule fibrillarin to avoid chemoresistance. Front Cell Dev Biol. 2018;6:1077. https://doi.org/10.3389/fcell.2018.01104.

5. Barria M, Pusic A, Zollo O, Costa M, Kondrashov N, Rego E, et al. Suppression of Myc oncogenic activity by ribosomal protein hpolinsufficiency. Nature. 2008;456:971–5. https://doi.org/10.1038/nature07449.

6. Barria M, Pusic A, Zollo O, Costa M, Kondrashov N, Rego E, et al. Suppression of Myc oncogenic activity by ribosomal protein hpolinsufficiency. Nature. 2008;456:971–5. https://doi.org/10.1038/nature07449.

7. Barria M, Pusic A, Zollo O, Costa M, Kondrashov N, Rego E, et al. Suppression of Myc oncogenic activity by ribosomal protein hpolinsufficiency. Nature. 2008;456:971–5. https://doi.org/10.1038/nature07449.

8. Barria M, Pusic A, Zollo O, Costa M, Kondrashov N, Rego E, et al. Suppression of Myc oncogenic activity by ribosomal protein hpolinsufficiency. Nature. 2008;456:971–5. https://doi.org/10.1038/nature07449.

9. Barria M, Pusic A, Zollo O, Costa M, Kondrashov N, Rego E, et al. Suppression of Myc oncogenic activity by ribosomal protein hpolinsufficiency. Nature. 2008;456:971–5. https://doi.org/10.1038/nature07449.

10. Barria M, Pusic A, Zollo O, Costa M, Kondrashov N, Rego E, et al. Suppression of Myc oncogenic activity by ribosomal protein hpolinsufficiency. Nature. 2008;456:971–5. https://doi.org/10.1038/nature07449.

11. Barria M, Pusic A, Zollo O, Costa M, Kondrashov N, Rego E, et al. Suppression of Myc oncogenic activity by ribosomal protein hpolinsufficiency. Nature. 2008;456:971–5. https://doi.org/10.1038/nature07449.

12. Barria M, Pusic A, Zollo O, Costa M, Kondrashov N, Rego E, et al. Suppression of Myc oncogenic activity by ribosomal protein hpolinsufficiency. Nature. 2008;456:971–5. https://doi.org/10.1038/nature07449.

13. Shubina MY, Musinova YR, Sheval EV. Proliferation, cancer, and aging-novel functions of the nuclear methyltransferase fibrillarin? Cell Biol Int. 2018;42:1463–6. https://doi.org/10.1002/cbi.11044.

14. El Hassouni B, Sarkisjan D, Vos JC, Giovannetti E, Peters GJ. Targeting the ribosome biogenesis key molecule fibrillarin to avoid chemoresistance. Curr Med Chem. 2018;26(33):6020–32. https://doi.org/10.2174/092967326668181203133332.

15. El Hassouni B, Sarkisjan D, Vos JC, Giovannetti E, Peters GJ. Targeting the ribosome biogenesis key molecule fibrillarin to avoid chemoresistance. Curr Med Chem. 2018;26(33):6020–32. https://doi.org/10.2174/092967326668181203133332.

16. Koh CM, Gurel B, Sultcliffe S, Ayye MJ, Schultz D, Iwata T, et al. Alterations in nucleolar structure and gene expression programs in prostatic neoplasia are driven by the MYC oncogene. Am J Pathol. 2011;178:1824–43. https://doi.org/10.1016/j.ajpath.2011.07.040.

17. Koh CM, Gurel B, Sultcliffe S, Ayye MJ, Schultz D, Iwata T, et al. Alterations in nucleolar structure and gene expression programs in prostatic neoplasia are driven by the MYC oncogene. Am J Pathol. 2011;178:1824–43. https://doi.org/10.1016/j.ajpath.2011.07.040.

18. Koh CM, Gurel B, Sultcliffe S, Ayye MJ, Schultz D, Iwata T, et al. Alterations in nucleolar structure and gene expression programs in prostatic neoplasia are driven by the MYC oncogene. Am J Pathol. 2011;178:1824–43. https://doi.org/10.1016/j.ajpath.2011.07.040.

19. Koh CM, Gurel B, Sultcliffe S, Ayye MJ, Schultz D, Iwata T, et al. Alterations in nucleolar structure and gene expression programs in prostatic neoplasia are driven by the MYC oncogene. Am J Pathol. 2011;178:1824–43. https://doi.org/10.1016/j.ajpath.2011.07.040.

20. Koh CM, Gurel B, Sultcliffe S, Ayye MJ, Schultz D, Iwata T, et al. Alterations in nucleolar structure and gene expression programs in prostatic neoplasia are driven by the MYC oncogene. Am J Pathol. 2011;178:1824–43. https://doi.org/10.1016/j.ajpath.2011.07.040.

21. Koh CM, Gurel B, Sultcliffe S, Ayye MJ, Schultz D, Iwata T, et al. Alterations in nucleolar structure and gene expression programs in prostatic neoplasia are driven by the MYC oncogene. Am J Pathol. 2011;178:1824–43. https://doi.org/10.1016/j.ajpath.2011.07.040.

22. Koh CM, Gurel B, Sultcliffe S, Ayye MJ, Schultz D, Iwata T, et al. Alterations in nucleolar structure and gene expression programs in prostatic neoplasia are driven by the MYC oncogene. Am J Pathol. 2011;178:1824–43. https://doi.org/10.1016/j.ajpath.2011.07.040.

23. Koh CM, Gurel B, Sultcliffe S, Ayye MJ, Schultz D, Iwata T, et al. Alterations in nucleolar structure and gene expression programs in prostatic neoplasia are driven by the MYC oncogene. Am J Pathol. 2011;178:1824–43. https://doi.org/10.1016/j.ajpath.2011.07.040.

24. Koh CM, Gurel B, Sultcliffe S, Ayye MJ, Schultz D, Iwata T, et al. Alterations in nucleolar structure and gene expression programs in prostatic neoplasia are driven by the MYC oncogene. Am J Pathol. 2011;178:1824–43. https://doi.org/10.1016/j.ajpath.2011.07.040.

25. Koh CM, Gurel B, Sultcliffe S, Ayye MJ, Schultz D, Iwata T, et al. Alterations in nucleolar structure and gene expression programs in prostatic neoplasia are driven by the MYC oncogene. Am J Pathol. 2011;178:1824–43. https://doi.org/10.1016/j.ajpath.2011.07.040.

26. Koh CM, Gurel B, Sultcliffe S, Ayye MJ, Schultz D, Iwata T, et al. Alterations in nucleolar structure and gene expression programs in prostatic neoplasia are driven by the MYC oncogene. Am J Pathol. 2011;178:1824–43. https://doi.org/10.1016/j.ajpath.2011.07.040.

27. Koh CM, Gurel B, Sultcliffe S, Ayye MJ, Schultz D, Iwata T, et al. Alterations in nucleolar structure and gene expression programs in prostatic neoplasia are driven by the MYC oncogene. Am J Pathol. 2011;178:1824–43. https://doi.org/10.1016/j.ajpath.2011.07.040.

28. Koh CM, Gurel B, Sultcliffe S, Ayye MJ, Schultz D, Iwata T, et al. Alterations in nucleolar structure and gene expression programs in prostatic neoplasia are driven by the MYC oncogene. Am J Pathol. 2011;178:1824–43. https://doi.org/10.1016/j.ajpath.2011.07.040.

29. Koh CM, Gurel B, Sultcliffe S, Ayye MJ, Schultz D, Iwata T, et al. Alterations in nucleolar structure and gene expression programs in prostatic neoplasia are driven by the MYC oncogene. Am J Pathol. 2011;178:1824–43. https://doi.org/10.1016/j.ajpath.2011.07.040.