Mitochondrial markers predict recurrence, metastasis and Tamoxifen-resistance in breast cancer patients: early detection of treatment failure with companion diagnostics

Sotgia, F, Fiorillo, M and Lisanti, MP

10.18632/oncotarget.19612

| Title | Mitochondrial markers predict recurrence, metastasis and Tamoxifen-resistance in breast cancer patients: early detection of treatment failure with companion diagnostics |
|---|---|
| Authors | Sotgia, F, Fiorillo, M and Lisanti, MP |
| Type | Article |
| URL | This version is available at: http://usir.salford.ac.uk/id/eprint/43484/ |
| Published Date | 2017 |

USIR is a digital collection of the research output of the University of Salford. Where copyright permits, full text material held in the repository is made freely available online and can be read, downloaded and copied for non-commercial private study or research purposes. Please check the manuscript for any further copyright restrictions.

For more information, including our policy and submission procedure, please contact the Repository Team at: usir@salford.ac.uk.
Mitochondrial markers predict recurrence, metastasis and Tamoxifen-resistance in breast cancer patients: Early detection of treatment failure with companion diagnostics

Federica Sotgia1, Marco Fiorillo1,2 and Michael P. Lisanti1
1 Translational Medicine, School of Environment & Life Sciences, Cockcroft Building, University of Salford, Greater Manchester, MS 4WT, United Kingdom
2 The Department of Pharmacy, Health and Nutritional Sciences, The University of Calabria, Cosenza, Italy

Correspondence to: Michael P. Lisanti, email: michaelp.lisanti@gmail.com
Correspondence to: Federica Sotgia, email: fsotgia@gmail.com

Keywords: mitochondria, mitochondrial biogenesis, biomarkers, treatment failure, relapse

Received: May 23, 2017 Accepted: June 16, 2017 Published: July 27, 2017

ABSTRACT

Here, we used a data-mining and informatics approach to discover new biomarkers of resistance to hormonal therapy in breast cancer. More specifically, we investigated whether nuclear-encoded genes associated with mitochondrial biogenesis can be used to predict tumor recurrence, distant metastasis and treatment failure in high-risk breast cancer patients. Overall, this strategy allowed us to directly provide in silico validation of the prognostic value of these mitochondrial components in large and clinically relevant patient populations, with >15 years of follow-up data. For this purpose, we employed a group of 145 ER(+) luminal A breast cancer patients, with lymph-node (LN) metastasis at diagnosis, that were treated with tamoxifen, but not any chemotherapy agents. Using this approach, we identified >60 new individual mitochondrial biomarkers that predicted treatment failure and tumor recurrence, with hazard-ratios (HR) of up to 4.17 (p=2.2e-07). These include mitochondrial chaperones (HSPD1, HSPA9), membrane proteins (VDAC2, TOMM70A) and anti-oxidants (SOD2), as well as 18 different mitochondrial ribosomal proteins (MRPs) and >20 distinct components of the OXPHOS complexes. In addition, we combined 4 mitochondrial proteins (HSPD1, UQCRB, MRPL15, COX17), to generate a compact mitochondrial gene signature, associated with a HR of 5.34 (p=1e-09). This signature also successfully predicted distant metastasis and was effective in larger groups of ER(+) (N=2,447), basal (N=540) and HER2(+) (N=193) breast cancers. It was also effective in all breast cancers (N=3,180), if considered together as a single group. Based on this analysis, we conclude that mitochondrial biogenesis should be considered as a new therapeutic target for overcoming tumor recurrence, distant metastasis and treatment failure in patients with breast cancer. In summary, we identified individual mitochondrial biomarkers and 2 compact mitochondrial gene signatures that can be used to predict tamoxifen-resistance and tumor recurrence, at their initial diagnosis, in patients with advanced breast cancer. In the long-term, these mitochondrial markers could provide a new companion diagnostics platform to help clinicians to accurately predict the response to hormonal therapy in ER(+) breast cancer patients, facilitating more personalized and effective treatment. Similarly, these mitochondrial markers could be used as companion diagnostics, to determine which breast cancer patients would benefit most from clinical treatments with mitochondrially-targeted anti-cancer therapeutics. Finally, we also showed that these mitochondrial markers are superior when directly compared with conventional biomarkers, such as Ki67 and PCNA.
INTRODUCTION

Treatment failure, due to drug resistance, still remains a major obstacle for more effective anti-cancer therapy and personalized medicine [1-9]. In estrogen-receptor-positive (ER(+) breast cancer, approximately 40-to-50% of patients eventually develop tamoxifen-resistance [5-9]. Importantly, the five-year survival rate following tamoxifen-resistance is less than 20% [1-5]. Unfortunately, tamoxifen-resistance often manifests itself as tumor recurrence and/or distant metastasis. As such, resistance to endocrine therapy is a critical factor that still limits the efficacy of breast cancer treatment. Thus, better biomarkers and companion diagnostics are needed for the early detection of patients that will likely fail hormonal therapy [5-9].

Here, we set out to test the hypothesis that individual markers of mitochondrial biogenesis and OXPHOS may have prognostic value in the early identification of tamoxifen-resistant patients at diagnosis, up to 15 years before the onset of tumor recurrence and distant metastasis. For this purpose, we performed outcome analysis on > 400 nuclear mitochondrial gene transcripts. Our results indicate that > 60 different mitochondrial markers can be used individually or in combination, as short signatures, to predict tumor recurrence in tamoxifen-treated breast cancer patients. As a consequence, we discuss the possibility that mitochondria should be therapeutically targeted, to overcome resistance to hormonal therapy, and to prevent tumor recurrence and distant metastasis. In accordance with this idea, metformin (a mitochondrial complex I inhibitor) has been previously shown to overcome tamoxifen-resistance in ER(+) cell culture models, which mimic the tumor microenvironment by the addition of stromal fibroblasts [9-11].

Interestingly, mitochondrial markers also showed prognostic value in different sub-groups of ER(-) breast cancer patients [12].

RESULTS

Establishing the prognostic value of conventional markers in the patient population

To identify new potential biomarkers of tamoxifen-resistance, here we used publically available transcriptional profiling data from the tumors of breast cancer patients that were treated with tamoxifen, but did not receive any chemotherapy. For this purpose, we selected high-risk patients that were lymph-node positive at diagnosis, and we focused on the luminal A subtype, which represents the most common form of ER(+) breast cancers (N = 145 patients) (Figure 1).

As proliferative markers are often used as the primary endpoint in clinical trials, we first assessed the prognostic value of Ki67 and PCNA, in this patient population. Table 1 and Figure 2A both show the
Figure 2: Conventional markers of proliferation and estrogen-receptor-alpha signaling predict clinical outcome in high-risk ER(+) breast cancer patients. We assessed the predictive value of Ki67 and PCNA in \( N = 145 \) ER(+) breast cancer patients, luminal A sub-type, that were lymph-node positive (LN(+)) at diagnosis, who were treated with tamoxifen and followed over a period of nearly 200 months (> 15 years). A. Note that high transcript levels of Ki67 and PCNA are associated with increased levels of tumor recurrence, indicative of tamoxifen-resistance. Please note that the official gene name for the Ki67 protein is MKI67. B. Note that high transcript levels of estrogen-receptor (ESR1) and cyclin D1 expression (CCND1) are both associated with reduced tumor recurrence, showing increased efficacy of tamoxifen therapy. RFS, recurrence or relapse free survival is shown (a.k.a., tumor recurrence).

Table 1: Prognostic value of known markers of proliferation

| Gene Probe ID | Symbol | Hazard-Ratio | Log-Rank Test |
|---------------|--------|--------------|---------------|
| 212022_s_at   | MKI67  | 2.52         | 0.002         |
| 217400_at     | PCNA   | 1.81         | 0.04          |

Table 2: Prognostic value of known markers of ER-signaling

| Gene Probe ID | Symbol | Hazard-Ratio | Log-Rank Test |
|---------------|--------|--------------|---------------|
| 205225_at     | ESR1   | 0.31         | 0.003         |
| 208711_s_at   | CCND1  | 0.53         | 0.025         |
| 200952_s_at   | CCND2  | 0.50         | 0.03          |
prognostic value of these markers. The hazard-ratios for Ki67 and PCNA were 2.5 and 1.8, respectively, for relapse-free survival (RFS) (i.e., tumor recurrence).

Next, we assessed the behavior of markers of estrogen receptor signaling in these patients. It would be predicted that increased levels of such markers would be associated with a positive response to hormonal therapy. As predicted, Table 2 and Figure 2B show that estrogen receptor-alpha (ESR1) and cyclin D1/2 levels (CCND1/2) both effectively predict tamoxifen-sensitivity, as reflected by a reduction in tumor recurrence.

Finally, we also assessed the prognostic value of two macrophage-specific markers of inflammation. Table 3 and Figure 3 show that CD68 and CD163 both effectively predict tumor recurrence, with hazard-ratios of 1.76 and 2.95, respectively.

Thus, conventional markers of proliferation, estrogen signaling, and inflammation can all be used to predict tumor-recurrence and tamoxifen-resistance in LN(+) luminal A breast cancer patients.

| Gene Probe ID | Symbol | Hazard-Ratio | Log-Rank Test |
|---------------|--------|--------------|---------------|
| 216233_at     | CD163  | 2.95         | 0.02          |
| 215049_x_at   | CD163  | 2.45         | 0.009         |
| 203645_s_at   | CD163  | 2.34         | 0.003         |
| 203507_at     | CD68   | 1.76         | 0.048         |
| Combined      |        | 2.31         | 0.003         |

Prognostic value of individual markers of mitochondrial biogenesis

To test our hypothesis that increased mitochondrial biogenesis contributes towards tumor recurrence and tamoxifen-resistance, we next assessed the prognostic value of specific mitochondrial markers.

First, we examined the behavior of mitochondrial chaperones (HSPs) and mitochondrial membrane proteins (TIMM/TOMM/VDAC families). Table 4 and Figure 4 show that HSP60 (HSPD1) and VDAC2 have the best prognostic value with hazard-ratios of 3.6 and 4.2, respectively. Importantly, several members of the TIMM and TOMM gene families also had prognostic value (HR = 1.8-to-2.8). AKAP1 and IMMT also had significant value (HR = 1.8-to-2.2). Notably, the mitochondrial anti-oxidant SOD2 also showed significant prognostic value, with a hazard-ratio of 2.94 (p = 0.0001) (Table 4). Similar results were obtained with mitochondrial creatine kinase isoforms (HR = 2.0-to-2.2).

Inflammation/Macrophages

Figure 3: Conventional markers of macrophage-associated inflammation predict poor clinical outcome in high-risk ER(+) breast cancer patients. Note that that high transcript levels of CD163 and CD68 are associated with increased levels of tumor recurrence and, therefore, tamoxifen-resistance.
Next, we examined the prognostic value of all the known mitochondrial ribosomal proteins (MRPs), which contribute to the protein translation of key members of the OXPHOS-related complexes, and are essential for mitochondrial biogenesis (summarized in Table 5).

Twelve different components of the large subunit (MRPLs) showed significant prognostic value, with hazard-ratios between 1.8 and 3.3. Most notably, MRPL15 had the best prognostic value (HR = 3.3; \( p = 1.6\text{e}{-05} \)).

Similarly, six different components of the small subunit (MRPSs) showed significant prognostic value, with hazard-ratios between 1.8 and 2.35.

Thus, 18 different MRPs all predicted tumor recurrence. Kaplan-Meier curves for representative examples are shown in Figure 5, panels A & B.

We also assessed the prognostic value of members of the OXPHOS complexes I-V. These results are summarized in Table 6. Remarkably, greater than 20.....
Figure 4: Mitochondrial chaperones and membrane proteins are associated with tumor recurrence in high-risk ER(+) breast cancer patients. Note that high transcript levels of HSPD1 and VDAC2 are associated with increased levels of tumor recurrence and resistance to hormonal therapy.

Figure 5: Mitochondrial ribosomal proteins (MRPs) are associated with tumor recurrence in high-risk ER(+) breast cancer patients. A. Note that high transcript levels of MRPL15 and MRPL18 predict increased tumor recurrence and tamoxifen-resistance. B. Similarly, high transcript levels of MRPS12 and MRPS11 predict increased tumor recurrence and tamoxifen-resistance.
Figure 6: Mitochondrial complex I and complex III proteins are associated with tumor recurrence in high-risk ER(+) breast cancer patients. A. Note that high levels of NDUFA8 and NDUFA6 predict increased tumor recurrence and tamoxifen-resistance. B. Similarly, high levels of UQCRB and UQCR6 predict increased tumor recurrence and tamoxifen-resistance.

Figure 7: Mitochondrial complex IV and complex V proteins are associated with tumor recurrence in high-risk ER(+) breast cancer patients. A. Note that high levels of COX17 and COX5B predict increased tumor recurrence and tamoxifen-resistance. B. Similarly, high levels of ATP5J and ATP5J2 predict increased tumor recurrence and tamoxifen-resistance.
Figure 8: A short mitochondrial signature (Mito-Signature-1) predicts poor clinical outcome in high-risk ER(+) breast cancer patients. Note that this short 4-gene signature (HSPD1/UQCRB/MRPL15/COX17) effectively predicts tumor recurrence and distant metastasis in LN(+) luminal A patients treated with tamoxifen therapy, indicative of treatment failure and tamoxifen-resistance.

Table 5: Prognostic value of mitochondrial Ribosomal proteins

| Gene Probe ID | Symbol | Hazard-Ratio | Log-Rank Test |
|---------------|--------|--------------|---------------|
| **Large Ribosomal Subunit** |
| 218027_at     | MRPL15 | 3.28         | 1.6e-05       |
| 217907_at     | MRPL18 | 2.91         | 0.0001        |
| 219244_s_at   | MRPL46 | 2.89         | 0.02          |
| 218270_at     | MRPL24 | 2.38         | 0.002         |
| 218049_s_at   | MRPL13 | 2.14         | 0.01          |
| 218281_at     | MRPL48 | 2.11         | 0.01          |
| 208787_at     | MRPL3  | 2.07         | 0.03          |
| 213897_s_at   | MRPL23 | 2.02         | 0.04          |
| 218105_s_at   | MRPL4  | 1.99         | 0.02          |
| 222216_s_at   | MRPL17 | 1.97         | 0.02          |
| 217919_s_at   | MRPL42 | 1.88         | 0.05          |
| 218202_x_at   | MRPL44 | 1.78         | 0.04          |
| **Small Ribosomal Subunit** |
| 204330_s_at   | MRPS12 | 2.35         | 0.03          |
| 211595_s_at   | MRPS11 | 2.26         | 0.01          |
| 219819_s_at   | MRPS28 | 1.88         | 0.03          |
| 217919_s_at   | MRPL42 | 1.88         | 0.05          |
| 219220_x_at   | MRPS22 | 1.85         | 0.04          |
| 218654_s_at   | MRPS33 | 1.84         | 0.04          |

Mito Signature 1

(HSPD1/UQCRB/MRPL15/COX17)
### Table 6: Prognostic value of mitochondrial OXPHOS complexes

| Gene Probe ID | Symbol  | Hazard-Ratio | Log-Rank Test |
|---------------|---------|--------------|---------------|
| Complex I     |         |              |               |
| 218160_at     | NDUFA8  | 2.45         | 0.002         |
| 202000_at     | NDUFA6  | 2.41         | 0.002         |
| 202001_s_at   | NDUFA6  | 2.23         | 0.006         |
| 203039_s_at   | NDUFS1  | 2.40         | 0.003         |
| 201740_at     | NDUFS3  | 2.17         | 0.006         |
| 203613_s_at   | NDUFB6  | 1.99         | 0.02          |
| 208714_at     | NDUFV1  | 1.96         | 0.03          |
| 203606_at     | NDUFS6  | 1.92         | 0.04          |
| 202298_at     | NDUFA1  | 1.89         | 0.03          |
| Complex III   |         |              |               |
| 209065_at     | UQCRB   | 3.42         | 1.9e-05       |
| 209066_x_at   | UQCRB   | 2.12         | 0.01          |
| 205849_s_at   | UQR6    | 2.53         | 0.002         |
| 201066_at     | UQR4    | 1.96         | 0.02          |
| 212600_s_at   | UQRC2   | 1.92         | 0.04          |
| Complex IV    |         |              |               |
| 203880_at     | COX17   | 2.99         | 7.6e-05       |
| 213735_s_at   | COX5B   | 2.51         | 0.001         |
| 202343_x_at   | COX5B   | 2.10         | 0.01          |
| 211025_x_at   | COX5B   | 2.08         | 0.01          |
| 202698_x_at   | COX4I1  | 2.36         | 0.02          |
| 200925_at     | COX6A1  | 2.14         | 0.01          |
| 218057_x_at   | COX4NB  | 1.99         | 0.04          |
| 217249_x_at   | COX7A2  | 1.90         | 0.03          |
| Complex V     |         |              |               |
| 202325_s_at   | ATP5J   | 2.65         | 0.01          |
| 202961_s_at   | ATP5J2  | 2.44         | 0.035         |
| 213366_x_at   | ATP5C1  | 2.19         | 0.01          |
| 208870_x_at   | ATP5C1  | 2.08         | 0.01          |
| 205711_x_at   | ATP5C1  | 2.00         | 0.02          |
| 217848_s_at   | PPA1    | 2.07         | 0.01          |
| 221677_s_at   | ATP5O   | 2.03         | 0.02          |
| 217801_at     | ATP5E   | 1.99         | 0.02          |
| 207508_at     | ATP5G3  | 1.93         | 0.02          |

### Table 7: Mito-signature 1 for predicting treatment failure

| Gene Probe ID | Symbol   | Hazard-Ratio | Log-Rank Test |
|---------------|----------|--------------|---------------|
| 200807_s_at   | HSPD1    | 3.61         | 5.9e-06       |
| 209065_at     | UQCRB    | 3.42         | 1.9e-05       |
| 218027_at     | MRPL15   | 3.28         | 1.6e-05       |
| 203880_at     | COX17    | 2.99         | 7.6e-05       |
| **Combined**  |          | **5.34**     | **1e-09**     |
different members of the OXPHOS complexes showed hazard-ratios between 1.9 and 3.4. UQCRB (complex III) had the best prognostic value (HR = 3.42; \( p = 1.9e-05 \)). Similarly, COX17 (complex IV) showed significant prognostic value (HR = 2.99; \( p = 7.6e-05 \)).

Kaplan-Meier curves for members of complex I and III are shown in Figure 6A & B, while results with members of complex IV and V are also shown in Figure 7A & 7B.

Two new mitochondrial gene signatures for predicting tumor recurrence, distant metastasis and tamoxifen-resistance

In order to increase the prognostic power of these individual mitochondrial biomarkers, we next selected the most promising ones and used them to create two new mitochondrial gene signatures. Mito-Signature-1 contains 4 genes (HSPD1, UQCRB, MRPL15, COX17), while Mito-Signature-2 consists of only 2 genes (HSPD1, VDAC2) (See Tables 7 & 8). K-M curves for these two signatures are shown in Figures 8 and 9.

Importantly, Mito-Signature-1 yielded a significantly improved hazard-ratio for tumor recurrence of 5.34 (\( p = 1e-09 \)). It was also highly predictive for distant metastasis, in the same group of patients (HR = 3.65; \( p = 4.9e-05 \)).

Similarly, Mito-Signature-2 showed a hazard-ratio for tumor recurrence of 5.2 (\( p = 6e-09 \)). Mito-Signature-2 was also highly predictive for distant metastasis (HR = 3.88; \( p = 6.8e-05 \)).

Thus, both mitochondrial signatures were a significant improvement over individual mitochondrial biomarkers, as well as Ki67, PCNA, ESR1, CCND1/2 and CD68/CD163 (compare with Figures 2 & 3).

**Table 8: Mito-signature 2 for predicting treatment failure**

| Gene Probe ID | Symbol | Hazard-Ratio | Log-Rank Test |
|---------------|--------|--------------|---------------|
| 211662_s_at   | VDAC2  | 4.17         | 2.2e-07       |
| 200807_s_at   | HSPD1  | 3.61         | 5.9e-06       |
| Combined      |        | 5.19         | 6e-09         |

Figure 9: A short mitochondrial signature (Mito-Signature-2) predicts poor clinical outcome in high-risk ER(+) breast cancer patients. Note that this short 2-gene signature (HSPD1/VDAC2) effectively predicts tumor recurrence and distant metastasis in LN(+) luminal A patients treated with tamoxifen therapy, indicative of treatment failure and tamoxifen-resistance.
Figure 10: Mitochondrial signatures 1 and 2 both have predictive value in a larger group of ER(+) breast cancer patients, who were treated with hormonal therapy. These patients were not subdivided into luminal A/B subgroups and were not sub-divided by lymph-node status. A. K-M analysis with Mito-Signatures 1 & 2, showing tumor recurrence. N = 698 patients. B. K-M analysis with Mito-Signatures 1 & 2, showing overall survival. N = 127 patients. C. K-M analysis with individual markers (HSPD1 and VDAC2) is also shown for comparison. N = 698 patients.
Figure 11: Mitochondrial signatures 1 and 2 both have predictive value in a larger group of ER(+) breast cancer patients, who were treated with hormonal therapy. These patients were not sub-divided into luminal A/B subgroups, but were sub-divided by lymph-node status (LN(+) versus LN(-)). A. K-M analysis with Mito-Signature-1 is shown for both groups: LN(+) (\(N = 221\) patients) and LN(-) (\(N = 403\) patients). B. K-M analysis with Mito-Signature-2 is shown for both groups: LN(+) (\(N = 221\) patients) and LN(-) (\(N = 403\) patients).

Figure 12: K-M analysis with conventional proliferative markers, in the same patient population, is shown for comparison. Note that Mito Signature 1 & 2 show better predictive value than both proliferative markers, namely KI67 and PCNA. A. K-M analysis with KI67 is shown for both groups: LN(+) (\(N = 221\) patients) and LN(-) (\(N = 403\) patients). B. K-M analysis with PCNA is shown for both groups: LN(+) (\(N = 221\) patients) and LN(-) (\(N = 403\) patients).
Two short mitochondrial gene signatures can effectively predict tumor recurrence in larger ER(+) patient populations that received hormonal therapy, as well as in ER(-) patients, and all breast cancers, considered as a single group.

We also examined the prognostic value of these two mitochondrial gene signatures in a larger group of ER(+) patients (N = 698), that received hormonal therapy, but not chemotherapy. This group of patients was not segregated into luminal A and luminal B subtypes.

Figure 10A shows the results of this K-M analysis for relapse-free survival: Mito-Signature-1 (HR = 2.65; \( p = 3.2e-11 \)) and Mitosignature-2 (HR = 3.3; \( p = 1.1e-16 \)). Similar results were also obtained for overall survival (Figure 10B).

Both of these mitochondrial signatures were also effective if the ER(+) patient population was divided into LN(+) and LN(-) groups (Figure 11A & 11B). In addition, both of these mitochondrial signatures were clearly superior to Ki67 and PCNA in this larger ER(+) patient population. However, Ki67 still showed prognostic value (Figure 12A), while PCNA had no prognostic value (Figure 12B).

Finally, we assessed the behavior of Mito-Signature-1 in even larger and more varied patient populations, where the therapy was not restricted to tamoxifen.

Supplemental Figure S1 shows that Mito-Signature-1 was also effective in ER(+) (N = 2,447), ER-/basal (N = 540), ER-/HER2(+) (N = 193), as well as in all breast cancer subtypes combined (N = 3,180). Similarly, Mito-Signature-1 was still statistically effective in both luminal A (N = 438 + 813) and luminal B (N = 907) patient populations (Supplemental Figure S2). Similarly, comparable results were obtained with Mito-Signature-2 (data not shown).

Thus, these mitochondrial-based gene signatures may represent important new prognostic tools for predicting patient outcomes, in a wide variety of different breast cancer patients, but especially in ER(+) patients treated with hormonal therapies.

**DISCUSSION**

Early detection of tamoxifen-resistance with mitochondrial markers: prevention of tumor recurrence and distant metastasis?

Here, we show that mitochondrial markers effectively predict tumor recurrence, distant metastasis and tamoxifen-resistance in high-risk ER(+) breast cancer patients. Importantly, these mitochondrial markers could now be used to identify high-risk ER(+) breast cancer patients at diagnosis, up to 15 years in advance, before they undergo tumor recurrence and metastasis. These results also suggest that mitochondria should be therapeutically-targeted in epithelial cancer cells to overcome tamoxifen-resistance and prevent the failure of hormonal therapy.

Consistent with this hypothesis, we have previously shown that treatment with metformin (a mitochondrial complex I inhibitor) is indeed sufficient to reverse tamoxifen-resistance in fibroblast-MCF7 co-cultures [10, 11]. Thus, targeting mitochondrial biogenesis and OXPHOS in ER(+) epithelial breast cancer cells may be a new therapeutic strategy for preventing or reversing tamoxifen-resistance in breast cancer patients.

Interestingly, these mitochondrial markers also showed predictive value in ER(-) breast cancer patients, both basal and HER2(+), suggesting that anti-mitochondrial therapy could be used as a more general anti-cancer strategy, against several different breast cancer sub-types.

A schematic diagram summarizing this new mito-based approach is presented in Figure 13. In this workflow, high-risk patients are first identified at diagnosis by the high expression of mitochondrial markers in their primary breast tumors. Then, these patients would be treated with mitochondrial-based therapeutics (e.g., metformin or another FDA-approved drug; in combination with the standard of care), to help prevent tumor recurrence and distant metastasis. Alternatively, novel mitochondrial-based...
based chemo-therapeutics could be developed against a variety of metabolic enzymes or structural proteins, to specifically target aggressive cancer cells with increased mitochondrial function.

**Evidence that mitochondrial power drives tamoxifen-resistance and cancer stem cell propagation**

Consistent with the above hypothesis, we recently showed that tamoxifen-resistant MCF7 cells (TAMR) are characterized by a metabolic phenotype, consisting of i) increased mitochondrial biogenesis, ii) increased ATP production and iii) reduced glutathione levels [13]. Thus, inhibition of mitochondrial function may be a new therapeutic strategy for overcoming tamoxifen-resistance in breast cancer patients. These findings could have important translational significance for the prevention of tumor recurrence in ER(+) breast cancers, which is due to an endocrine resistance phenotype [13]. Importantly, mitochondrial proteins may represent i) new prognostic biomarkers, ii) novel therapeutic targets and iii) companion diagnostics, for predicting and overcoming tamoxifen-resistance in different subsets of ER(+) breast cancer patients.

Similarly, based on high-resolution proteomics analysis, we have also proposed that mitochondrial biogenesis is an important driver of the cancer stem cell (CSC) phenotype [14, 15]. A key correlate of this assertion is that high mitochondrial mass is a metabolic biomarker for CSCs. To directly test this idea experimentally, we used a fluorescent dye, known as MitoTracker, to detect and quantitate mitochondrial mass in ER(+) breast cancer cells (MCF7) [16]. Using this approach, we purified the Mito-high and the Mito-low cell populations by flow cytometry (FACS). Remarkably, the Mito-high cell population was clearly enriched in cells with the characteristics of CSCs. Virtually identical results were also obtained with MDA-MB-231 cells, an ER(-) cell line. Thus, the use of “metabolic fractionation”, employing mitochondrial-based probes and flow cytometry, could be a successful new approach to the functional purification of drug-resistant CSCs. In accordance with this hypothesis, Mito-high breast cancer cells were also resistant to DNA-damage induced by Paclitaxel [16]. Thus, mitochondrial mass and function are directly linked to i) the CSC phenotype and ii) chemotherapeutic drug resistance, as well as iii) resistance to anti-estrogen therapy [13-24]. As such, we conclude that the association we observed here of high levels of mitochondrial markers (mRNA species and/or protein products) with poor clinical outcome in breast cancer patients may functionally reflect the presence of drug-resistant CSCs, driving tumor recurrence, metastasis and treatment failure.

**Using mitochondrial markers as companion diagnostics for drug re-purposing, treatment stratification and new drug discovery**

Several classes of FDA-approved antibiotics safely inhibit either mitochondrial biogenesis or OXPHOS as off-target “side-effects”. These include the tetracyclines (doxycycline), the erythromycins (azithromycin), pyrvinium pamoate, atovaquone, and bedaquiline, among others [13-24]. Therefore, the new mitochondrial biomarkers that we identified here could be used in combination with these FDA-approved drugs, as companion diagnostics. This would allow clinicians to select the right patient populations for new clinical trials aimed at drug re-purposing/re-positioning, for the prevention of tumor recurrence in ER(+) patients receiving anti-endocrine therapy.

Importantly, the novel mitochondrial biomarkers that we identified here may also be new therapeutic targets for future drug development aimed at combating the emergence of resistance to hormonal therapy. Based on our K-M analysis, the mitochondrial ribosome (a.k.a., mitoribosome) and its individual subunits would be attractive targets for intervention; in addition, mitochondrial chaperones, the OXPHOS complexes (I-IV) and the mitochondrial ATP-synthase (complex V) may also be tractable targets. Since several members of each of these multi-subunit complexes show prognostic value, this provides an indication that inactivation, or specific modulation, of the activity of each of these complexes may provide significant therapeutic benefits. Therapeutic targeting of these complexes would be expected to prevent tumor recurrence and distant metastasis, as well as confer tamoxifen-sensitivity, in ER(+) breast cancer patients.

**MATERIALS AND METHODS**

**Kaplan-Meier (K-M) analyses**

To perform K-M analysis on > 400 nuclear mitochondrial gene transcripts, we used an open-access online survival analysis tool to interrogate publically available microarray data from up to 3,455 breast cancer patients [12]. This allowed us to determine their prognostic value. For this purpose, we primarily analyzed data from ER(+) patients that were LN(+) at diagnosis and were of the luminal A sub-type, that were primarily treated with tamoxifen and not other chemotherapy (N = 145 patients). In this group, 100% the patients received some form of hormonal therapy and ~95% of them received tamoxifen. Biased and outlier array data were excluded from the analysis. This allowed us to identify > 60 nuclear mitochondrial gene transcripts, with significant prognostic value. Hazard-ratios were calculated, at the best

www.impactjournals.com/oncotarget 14  Oncotarget
auto-selected cut-off, and p-values were calculated using the logrank test and plotted in R. K-M curves were also generated online using the K-M-plotter (as high-resolution TIFF files), using univariate analysis:

http://kmplot.com/analysis/index.php?p = service&cancer = breast

This allowed us to directly perform in silico validation of these mitochondrial biomarker candidates. The multi-gene classifier function of the program was used to test the prognostic value of short mitochondrial gene signatures, using the mean expression of the selected probes. The 2012 version of the database was originally utilized for all these analyses, because a higher percentage of the patients used tamoxifen; however, virtually identical results were also obtained with the 2014 and 2017 versions.

Abbreviations

CSCs, cancer stem-like cells; DMFS, distant metastasis-free survival; ER, estrogen receptor alpha (ESR1); HR, hazard ratio; K-M, Kaplan-Meier; LN, lymph node; MRPL, mitochondrial ribosomal proteins, large subunit; MRPS, mitochondrial ribosomal proteins, small subunit; N, number of patients in a given data set; OXPHOS, oxidative phosphorylation (mitochondrial respiration); RFS, recurrence- or relapse-free survival

Author contributions

Professor Lisanti and Dr. Sotgia conceived and initiated this project. Professor Lisanti and Dr. Sotgia performed the bioinformatics analysis, and wrote the first draft of the manuscript, which was then further edited by Marco Fiorillo.

ACKNOWLEDGMENTS

It should be noted that this bioinformatics analysis, focused on nuclear-encoded mitochondrial-related gene transcripts, was not funded by a specific grant and did not require any research expenditures, since no “wet” laboratory experiments were performed.

CONFLICTS OF INTEREST

MPL and FS hold a minority interest in Lunella, Inc.

REFERENCES

1. C.K. Osborne and S.A. Fuqua. Mechanisms of tamoxifen resistance. Breast Cancer Res Treat. 1994;32: 49-55.
2. A. Ring and M. Dowsett. Mechanisms of tamoxifen resistance. Endocr Relat Cancer. 2004;11: 643-58.
3. S. Aliand and R.C. Coombes. Endocrine-responsive breast cancer and strategies for combating resistance. Nature Reviews Cancer. 2002; 2:101-12.
4. V.C. Jordan. Selective estrogen receptor modulation: concept and consequences in cancer. Cancer Cell. 2004; 5:207-13.
5. X. Li, M.T. Lewis, J. Huang, C. Gutierrez, C.K. Osborne, M.F. Wu, S.G. Hilsenbeck, A. Pavlick, X. Zhang, G.C. Channess, H. Wong, J. Rosen, and J.C. Chang. Intrinsic resistance of tumorigenic breast cancer cells to chemotherapy. Journal of the National Cancer Institute. 2008; 100: 672-79.
6. M. Giuliano, R. Schifp, C.K. Osborne, and M.V. Trivedi. Biological mechanisms and clinical implications of endocrine resistance in breast cancer. Breast. 2011; 20 Suppl 3: S42-49.
7. C.K. Osborneand R. Schiff. Mechanisms of endocrine resistance in breast cancer. Annual Review of Medicine. 2011; 62: 233-47.
8. R. Clarke, J.J. Tyson, and J.M. Dixon. Endocrine resistance in breast cancer—An overview and update. Molecular and Cellular Endocrinology. 2015; 418 Pt 3:220-34.
9. J. Dittmerand B. Leyh. The impact of tumor stroma on drug response in breast cancer. Seminars in Cancer Biology. 2015; 31:3-15.
10. U.E. Martinez-Outschoorn, A. Goldberg, Z. Lin, Y.H. Ko, N. Flomenberg, C. Wang, S. Pavlides, R.G. Pestell, A. Howell, F. Sotgia, and M.P. Lisanti. Anti-estrogen resistance in breast cancer is induced by the tumor microenvironment and can be overcome by inhibiting mitochondrial function in epithelial cancer cells. Cancer Biology & Therapy. 2011; 12:924-38.
11. U.E. Martinez-Outschoorn, Z. Lin, Y.H. Ko, A.F. Goldberg, N. Flomenberg, C. Wang, S. Pavlides, P.G. Pestell, A. Howell, F. Sotgia and M.P. Lisanti. Understanding the metabolic basis of drug resistance: Therapeutic induction of the Warburg effect kills cancer cells. Cell Cycle. 2011; 10: 2521-8.
12. B. Gyorffy, A Lanczky, AC Eklund, C Denkert, J Budczies, Q Li, Z Szallasi. An online survival analysis tool to rapidly assess the effect of 22,277 genes on breast cancer prognosis using microarray data of 1809 patients, Breast Cancer Res Treatment, 2010; 123: 725-31.
13 M. Fiorillo, F. Sotgia, D. Sisci, A.R. Cappello and M.P. Lisanti. Mitochondrial “power” drives tamoxifen resistance: NQO1 and GCLC are new therapeutic targets in breast cancer. Oncotarget, 2017; 8: 20309-327.
14. R. Lamb, B. Ozsvari, CL Lisanti, H.B. Tanowitz, A. Howell, U.E. Martinez-Outschoorn, F. Sotgia, M.P. Lisanti.
Antibiotics that target mitochondria effectively eradicate cancer stem cells, across multiple tumor types: treating cancer like an infectious disease. Oncotarget. 2015; 6: 4569-84.

16. G. Farnie, F. Sotgia, M.P. Lisanti. High mitochondrial mass identifies a sub-population of stem-like cancer cells that are chemo-resistant. Oncotarget. 2015; 6: 30472-86.

17. M. Fiorillo, R. Lamb, H.B. Tanowitz, L. Mutti, M. Krstic-Demonacos, A.R. Cappello, U.E. Martinez-Outschoorn, F. Sotgia, and M.P. Lisanti. Repurposing atovaquone: targeting mitochondrial complex III and OXPHOS to eradicate cancer stem cells. Oncotarget. 2016; 7:34084-99.

18. A. De Luca, M. Fiorillo, M. Peiris-Pages, B. Ozsvari, D.L. Smith, R. Sanchez-Alvarez, U.E. Martinez-Outschoorn, A.R. Cappello, V. Pezzi, M.P. Lisanti, and F. Sotgia. Mitochondrial biogenesis is required for the anchorage-independent survival and propagation of stem-like cancer cells. Oncotarget. 2015; 6:14777-95.

19. M. Fiorillo, R. Lamb, H.B. Tanowitz, A.R. Cappello, U.E. Martinez-Outschoorn, F. Sotgia, and M.P. Lisanti. Bedaquiline, an FDA-approved antibiotic, inhibits mitochondrial function and potently blocks the proliferative expansion of stem-like cancer cells (CSCs). Aging. 2016; 8:1593-607.

20. U.E. Martinez-Outschoorn, M. Peiris-Pages, R.G. Pestell, F. Sotgia, and M.P. Lisanti. Cancer metabolism: a therapeutic perspective. Nature Reviews Clinical Oncology. 2017; 14: 11-31.

21. R. Lamb, M. Fiorillo, A. Chadwick, B. Ozsvari, K.J. Reeves, D.L. Smith, R.B. Clarke, S.J. Howell, A.R. Cappello, U.E. Martinez-Outschoorn, M. Peiris-Pages, F. Sotgia, and M.P. Lisanti. Doxycycline down-regulates DNA-PK and radiosensitizes tumor initiating cells: Implications for more effective radiation therapy. Oncotarget. 2015; 6:14005-25.

22. G. Bonuccelli, M. Peiris-Pages, B. Ozsvari, U.E. Martinez-Outschoorn, F. Sotgia, M.P. Lisanti. Targeting cancer stem cell propagation with palbociclib, a CDK4/6 inhibitor: Telomerase drives tumor cell heterogeneity. Oncotarget. 2017; 8: 9868-84.

23. M. Peiris-Pagès, U.E. Martinez-Outschoorn, R.G. Pestell, F. Sotgia, M.P. Lisanti. Cancer stem cell metabolism. Breast Cancer Res. 2016; 18: 55.

24. G. Bonuccelli, E.M. De Francesco, M.A.G. Rianne de Boer, H.B. Tanowitz and M.P. Lisanti. NADH autofluorescence, a new metabolic biomarker for cancer stem cells: Identification of Vitamin C and CAPE as natural products targeting “stemness”. Oncotarget, 2017; 8: 20667-78.