High mitochondrial content is associated with breast cancer aggressiveness

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Abstract. Mitochondria are relevant for cancer initiation and progression. Antibodies against mitochondrially encoded cytochrome c oxidase II (MTCO2), targeting a mitochondria specific epitope, can be used to quantitate the mitochondria content of tumor cells. The present study evaluated the impact of the cellular mitochondrial content on the prognosis of patients with breast cancer using immunohistochemical analysis on 2,197 arrayed breast cancer specimens. Results were compared with histological tumor parameters, patient overall survival, tumor cell proliferation using Ki67 labeling index (Ki67LI) and various other molecular features. Tumor cells exhibited stronger MTCO2 expression than normal breast epithelial cells. MTCO2 immunostaining was largely absent in normal breast epithelium, but was observed in 71.9% of 1,797 analyzable cancer specimens, including 34.6% tumors with weak expression, 22.3% with moderate expression and 15.0% with strong expression. High MTCO2 expression was significantly associated with advanced tumor stage, high Bloom-Richardson-Elston/Nottingham (BRE) grade, nodal metastasis and shorter overall survival (P<0.0001 each). In multivariate analysis, MTCO2 expression did not provide prognostic information independent of BRE grade, pathological tumor and pathological lymph node status. Additionally, significant associations were observed for high MTCO2 expression and various molecular features, including high Ki67LI, amplifications of HER2, MYC, CCND1 and MDM2, deletions of PTEN, 8p21 and 9p, low estrogen receptor expression (P<0.0001 each) and progesterone receptor expression (P<0.0001). The present study demonstrated that high MTCO2 expression was strongly associated with a poor prognosis and unfavorable phenotypical and molecular tumor features in patients with breast cancer. This suggests that the mitochondrial content may have a pivotal role in breast cancer progression.

Introduction

Breast cancer, the most common malignancy in women (1), is treated by surgical removal of the cancer. In addition, adjuvant systemic therapy is given depending on the perceived aggressiveness of the removed cancer. Currently the established prognostic parameter include histological grade, tumor size, presence of lymph node metastasis, tumor cell proliferation (Ki67 labeling index; Ki67LI) as well as hormonal receptor and HER2 status (2-4) (Ki67) (5). In many patients, supplementary molecular parameters are analyzed (6-8). These molecular classifiers are built on multiplexed analysis of the mRNAs of 21-70 genes (9-11).

The rising interest in mitochondrial function and dysfunction on cancer development has been reviewed by Davis and Williams and Hsu et al (12,13). The loss of proliferation control in cancer cells may result in cellular...
masses that extend beyond the capacity of the supporting vasculature, leading to oxygen and nutrient deprivation. Hence, tumor cells must adapt to overcome these restrictions. Mitochondria are key organelles for energy production in normal and neoplastic cells. Quantity and activity of mitochondria are essential for tumor growth (reviewed in refs. 12-16). Mutations in mitochondrial genes or aberrant mitochondrial content have been described to occur in various cancer types (17-20). An increased mitochondria quantity has earlier been linked to aggressive tumor phenotype and poor prognosis in lung (21), colorectal (22,23), prostate (24), gastric (25), cervical (18), and ovarian cancer (26). In glioma, however, high mitochondria content was linked to favorable prognosis (27). In one study, on 76 breast carcinomas, a prognostic impact of the mitochondria count was also suggested (28). Focused on these reports, we assumed that the cellular mitochondria content of breast cancer cells might potentially be clinically relevant in breast cancer.

The mitochondrial encoded cytochrome c oxidase II (MTCO2) monoclonal antibody recognizes a 60 kDa non-glycosylated protein subunit of cytochrome c oxidase in mitochondria found in human cells and has been used to reveal the mitochondrial content of tumor cells in previous studies (24,28,29). We tested the clinical relevance of the cellular mitochondria content in breast cancer on a pre-existing breast cancer tissue microarray (TMA) containing more than 2,000 cancers. The data show that a ‘mitochondrion-rich phenotype’ represents a strong and independent predictor of patient prognosis in breast cancer.

Materials and methods

Patients. A total of 2,197 human breast cancer samples from paraffin-embedded tissue specimens fixed in 4% neutral buffered formalin were used (30). The breast cancer samples were consecutively collected between 1984 and 2000 and follow-up data were retrospectively collected. The median patient's age was 63 (range, 25-101) years. Overall survival data were available from 1,982 patients (713 patients with and 1,508 without event). The mean follow-up time was 63 months (range, 1-176 months). The TMA was produced as a TMA was produced as an adhesive coated plate containing more than 2,000 cancers. The data show that a ‘mitochondrion-rich phenotype’ represents a strong and independent predictor of patient prognosis in breast cancer.

Results

Technical issues. A total of 1,797 (81.8%) of the 2,197 arrayed tumor samples were interpretable in our TMA analysis. Non-informative cases (400 spots; 18.2%) were due to missing tissue samples or the absence of unequivocal cancer tissue in the TMA spot.

MTCO2 immunostaining in normal breast tissue and breast cancer. There were 20 normal breast tissue samples included in our TMA. Normal breast tissues showed negative to moderate MTCO2 staining in luminal cells under the chosen experimental conditions. In cancer, MTCO2 immunostaining was considered weak in 34.6%, moderate in 22.3% and strong in 15.0% of tumors. A total of 506 (28.2%) showed no detectable MTCO2 staining and were categorized as negative. Characteristic images of MTCO2 immunostainings are shown in Fig. 1. The intensity of MTCO2 immunostaining varied between histological breast cancer subtypes (Table I). Strong MTCO2 staining was significantly more common in medullary (27.9%), papillary (16.0%) and cancers of no special type (NST; 16.6%) than in lobular (6.9%) or tubular carcinomas (4.9%). Strong MTCO2 staining was also commonly seen in some of the rare breast cancer subtypes such as in 3 of 13 carcinomas with apocrine differentiation, 17 of 61 carcinomas with medullary features and 2 of 12 glycogen-rich clear cell type carcinomas (Table S1).

Association with tumor phenotype and molecular features. High levels of MTCO2 immunostaining were significantly associated with high pT stage, high BRE grade, estrogen and progesterone receptor negativity as well as HER2 overexpression or amplification (P<0.0001 each, Tables I and II). This was also seen for NST carcinomas (P<0.01, Table I). Further analyses with previously described frequent and prognostic relevant molecular features of breast cancers such as HER2 (35), and c-MYC amplification (32) as well as deletions of 8p21 (34), 9p21 (33), and 10q23 (36) showed...
significant associations with high MTCO2 staining intensity (Table II).

**Association with tumor cell proliferation.** Data on tumor cell proliferation as evaluated by the Ki67LI were available from a previous study with the same TMA (30). The mean Ki67LI increased from 19.62±0.66 in MTCO2 negative cancers to 37.75±0.93 in cancers with strong MTCO2 staining (P<0.0001). This statistically significant relationship was also seen in tumor subsets with identical pT or pN stage, lobular and medullary carcinoma, BRE grade and HER2 status as well as 8p and PTEN deletion. All data are summarized in Table III.

**Prognostic significance of MTCO2 expression.** Survival data were available for 1,806 cancers with interpretable IHC results. The rate of surviving patients continuously decreased with increasing levels of MTCO2 immunostaining (P=0.0001; Fig. 2). The association between strong MTCO2 immunostaining and poor prognosis was also seen in the subgroup of NST cancers (P<0.0001; Fig. 2) and in the nodal positive subset (P<0.0001; Fig. 2) and to a much lesser extent...
Table II. Association between MTCO2 staining and molecular alterations.

| Molecular alterations | N   | Negative, % | Weak, % | Moderate, % | Strong, % | P-value |
|-----------------------|-----|-------------|---------|-------------|-----------|---------|
| HER2 normal           | 1,141 | 29.2       | 36.3    | 21.2       | 13.3      | <0.0001 |
| HER2 amplified        | 239  | 15.5       | 32.6    | 30.1       | 21.8      |         |
| MYC normal            | 1,232 | 26.9       | 34.9    | 23.1       | 15.1      | <0.0001 |
| MYC amplified         | 64   | 7.8        | 29.7    | 28.1       | 34.4      |         |
| 8p21 normal           | 578  | 27.7       | 39.1    | 20.9       | 12.3      | <0.0001 |
| 8p21 deletion         | 553  | 17.0       | 27.7    | 29.5       | 25.9      |         |
| 9p21 normal           | 835  | 25.0       | 33.1    | 24.4       | 17.5      | 0.0182  |
| 9p21 deletion         | 150  | 16.7       | 28.0    | 32.0       | 23.3      |         |
| 10q23 normal          | 904  | 25.0       | 35.0    | 22.9       | 17.1      | <0.0001 |
| 10q23 deletion        | 216  | 11.6       | 26.4    | 36.1       | 25.9      |         |

MTCO2, mitochondrially encoded cytochrome c oxidase II.

Figure 1. Representative images of MTCO2 staining in breast cancer tissues. (A) Normal breast tissue, (B) negative staining in breast cancer tissue, (C) weak staining in breast cancer tissue, (D) moderate staining in breast cancer tissue and (E) strong staining in breast cancer tissue. Scale bar, 100 µm. MTCO2, mitochondrially encoded cytochrome c oxidase II.
### Table III. Association between MTCO2 staining and Ki67LI.

| Cases            | MTCO2 staining | N   | Ki67LI      | P-value |
|------------------|----------------|-----|-------------|---------|
| All cases        | Negative       | 428 | 19.6±0.7    | <0.0001 |
|                  | Weak           | 523 | 27.0±0.6    |         |
|                  | Moderate       | 338 | 33.0±0.8    |         |
|                  | Strong         | 216 | 37.8±0.9    |         |
| No special type  | Negative       | 264 | 20.7±0.8    | <0.0001 |
|                  | Weak           | 383 | 27.8±0.7    |         |
|                  | Moderate       | 253 | 33.3±0.9    |         |
|                  | Strong         | 168 | 38.0±1.1    |         |
| Lobular cancer   | Negative       | 92  | 16.2±1.2    | <0.0001 |
|                  | Weak           | 66  | 20.3±1.4    |         |
|                  | Moderate       | 24  | 28.4±2.3    |         |
|                  | Strong         | 15  | 26.9±2.9    |         |
| Medullary cancer | Negative       | 9   | 29.9±5.2    | 0.0109  |
|                  | Weak           | 15  | 43.7±4.1    |         |
|                  | Moderate       | 16  | 50.2±3.9    |         |
|                  | Strong         | 15  | 50.9±4.0    |         |
| HER2 amplified   | Negative       | 32  | 26.7±2.3    | <0.0001 |
|                  | Weak           | 67  | 34.2±1.6    |         |
|                  | Moderate       | 64  | 40.3±1.6    |         |
|                  | Strong         | 43  | 41.3±1.9    |         |
| MYC amplified    | Negative       | 4   | 28.5±7.4    | 0.3927  |
|                  | Weak           | 19  | 38.3±3.4    |         |
|                  | Moderate       | 17  | 41.6±3.6    |         |
|                  | Strong         | 21  | 41.6±3.2    |         |
| 8p deletion      | Negative       | 86  | 24.8±1.5    | <0.0001 |
|                  | Weak           | 135 | 30.2±1.2    |         |
|                  | Moderate       | 145 | 35.3±1.2    |         |
|                  | Strong         | 116 | 40.3±1.3    |         |
| PTEN deletion    | Negative       | 24  | 30.6±3.2    | 0.0118  |
|                  | Weak           | 55  | 37.7±2.1    |         |
|                  | Moderate       | 75  | 41.7±1.8    |         |
|                  | Strong         | 44  | 42.2±2.3    |         |
| pT1              | Negative       | 192 | 19.0±0.9    | <0.0001 |
|                  | Weak           | 200 | 23.8±0.9    |         |
|                  | Moderate       | 90  | 29.9±1.3    |         |
|                  | Strong         | 31  | 37.8±2.3    |         |
| pT2              | Negative       | 170 | 19.9±1.1    | <0.0001 |
|                  | Weak           | 238 | 29.6±0.9    |         |
|                  | Moderate       | 179 | 35.3±1.1    |         |
|                  | Strong         | 127 | 37.9±1.3    |         |
| pT3              | Negative       | 23  | 18.2±3.1    | <0.0001 |
|                  | Weak           | 27  | 31.2±2.9    |         |
|                  | Moderate       | 22  | 30.1±3.2    |         |
|                  | Strong         | 16  | 43.8±3.7    |         |
| pT4              | Negative       | 41  | 21.9±2.2    | <0.0001 |
|                  | Weak           | 57  | 25.3±1.7    |         |
|                  | Moderate       | 44  | 31.9±1.9    |         |
|                  | Strong         | 41  | 34.9±2.1    |         |
| BRE G1           | Negative       | 150 | 15.5±0.8    | <0.0001 |
|                  | Weak           | 127 | 19.5±0.9    |         |
|                  | Moderate       | 45  | 21.4±1.5    |         |
|                  | Strong         | 25  | 26.4±1.9    |         |
| BRE G2           | Negative       | 170 | 18.8±0.9    | <0.0001 |
|                  | Weak           | 208 | 23.7±0.8    |         |
|                  | Moderate       | 134 | 28.9±0.1    |         |
|                  | Strong         | 63  | 31.4±1.4    |         |
also in nodal negative NST cancers (P=0.0418; Fig. 2). Multivariate analysis for NST cancers including pT stage, nodal status, and BRE grade did not identify MTCO2 immunostaining as an independent prognosticator of survival, however (Table IV).

Discussion

Our study shows that high mitochondria content is significantly linked to disadvantageous tumor phenotype and bad prognosis in breast cancer.
Mitochondria (Mt) are abundant organelles in every cell, and their quantity is regulated by mitochondrial biogenesis and programmed cell death (mitophagy) (48-50). The transcription factor c-Myc is best known for its critical role in cell cycle regulation, cell growth, metabolism, and apoptosis (41-43). However, c-Myc also targets more than 400 different mitochondrial genes (38-41,44). Studies have demonstrated that an elevated or reduced c-Myc protein quantity leads to an increased/diminished mitochondrial mass (45,46). This couples c-Myc's role of a key activator of cell cycle activity with mitochondrial biogenesis. As such, c-Myc increases cellular biosynthetic and respiratory capacity by upregulating mitochondrial metabolism to complement its effects on stimulating cell cycle progression to coordinate rapid cell growth (45,47).

A critical role of high mitochondrion count for cell proliferation in breast cancer is supported by our data showing a striking link between MTCO2 expression and a high Ki67LI which was also visible in the vast majority of groups defined by identical morphological or molecular features. The prominent association found between c-Myc amplification and high MTCO2 expression fits well with the key role of c-Myc as an activator of mitochondrial biogenesis in cancer (38-40). The transcription factor c-Myc is best known for its critical role in cell cycle regulation, cell growth, metabolism, and apoptosis (41-43). However, c-Myc also targets more than 400 different mitochondrial genes (38-41,44). Studies have demonstrated that an elevated or reduced c-Myc protein quantity leads to an increased/diminished mitochondrial mass (45,46). This couples c-Myc's role of a key activator of cell cycle activity with mitochondrial biogenesis. As such, c-Myc increases cellular biosynthetic and respiratory capacity by upregulating mitochondrial metabolism to complement its effects on stimulating cell cycle progression to coordinate rapid cell growth (45,47).

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The PTEN-induced putative kinase 1 (PINK1)/Parkin pathway is a major inducer of mitophagy. It is triggered by mitochondrial membrane depolarization, a signal of mitochondrial dysfunction that results from lack of reducing equivalents, hypoxia and impaired electron transport [reviewed in (48)]. The conspicuous relationship between PTEN deletion and high MTCO2 staining in our study may thus indicate that high mitochondria quantities may also be caused by reduced mitophagy. Although clearance of damaged mitochondria via mitophagy is viewed to be also critical for cellular fitness since dysfunctional mitochondria can impair the electron transport chain function, reduced mitophagy can thus indicate that high mitochondria quantities may also be associated with mitochondrially encoded cytochrome c oxidase II (2). The higher level of MTCO2 immunostaining is highly specific for the mitochondrial DNA encoded second subunit of cytochrome c oxidase and can thus be used to quantitate the mitochondria content by IHC (29). Although mitochondria are present in every normal and neoplastic human cell, 28.2% of our tumors had a negative staining result. This was due to our approach to define experimental conditions, which distinguish cancers with low and high mitochondria quantities. The higher level of MTCO2 immunostaining in breast cancers as compared to normal breast tissues fits with the concept that neoplastic transformation goes along with higher cellular activity requiring more active mitochondria. That a striking further increase of MTCO2 immunostaining was detected with rising tumor grade and stage, demonstrates that elevated numbers of mitochondria are also supporting cancer progression. This is consistent with increasing energy requirement and a rearranged metabolism during tumor progression. Our data fit well with findings in multiple other cancer types, including lung (21), colorectal (22,23), prostate (24), gastric (25), cervical (18), and ovarian cancer (26), where a similar link between high levels of MTCO2 with adverse tumor phenotype and bad prognosis was shown.

In this study, a ubiquitously expressed protein was quantitated by IHC. The TMA approach is optimal for the identification of subtle staining differences of proteins that are abundantly present in cancer, such as mitochondrial components, because TMAs enable maximal experimental standardization at all levels. In our study, more than 1,700 breast cancers were analyzed the same day for maximal standardization. Moreover, all TMA sections were cut on one day immediately before staining in order to avoid unequal decay of a tissues reactivity to antibody binding (37). Finally, one pathologist interpreted all immunostainings in one continuous session to enable maximal standardization of staining interpretation. In earlier studies, this breast cancer TMA enabled us to validate the prognostic impact of several well-established prognostic biomarkers, such as HER2 alterations, estrogen and progesterone receptor expression (30), high Ki67LI, nuclear p53 accumulation (30), and PTEN deletion (34). These earlier data demonstrate the utility of our patient cohort to identify prognostic biomarkers.

The molecular database that has been collected during earlier studies for our set of cancers offers the advantage that biomarkers of interest can always be compared with preexisting data. For the purpose of this study, we had selected HER2 amplification as well as estrogen and progesterone receptor expression because of their central role in breast cancer. The strong link between MTCO2 expression and these important features further illustrates the importance of the mitochondria quantity in breast cancer. Our analyses also included Ki67LI as another pivotal parameter for cellular activity and various further chromosomal deletions and amplifications because of the role of some of them for regulating mitochondrial homeostasis.

Mitochondrial homeostasis is critical for cancer. A sufficiently high production of mitochondria is required to suffice the needs for energy production and cell metabolism. The prominent association found between c-Myc amplification and high MTCO2 expression fits well with the key role of c-Myc as an activator of mitochondrial biogenesis in cancer (38-40). The transcription factor c-Myc is best known for its critical role in cell cycle regulation, cell growth, metabolism, and apoptosis (41-43). However, c-Myc also targets more than 400 different mitochondrial genes (38-41,44). Studies have demonstrated that an elevated or reduced c-Myc protein quantity leads to an increased/diminished mitochondrial mass (45,46). This couples c-Myc's role of a key activator of cell cycle activity with mitochondrial biogenesis. As such, c-Myc increases cellular biosynthetic and respiratory capacity by upregulating mitochondrial metabolism to complement its effects on stimulating cell cycle progression to coordinate rapid cell growth (45,47).

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Decreased mitophagy may allow for a permissive threshold of dysfunctional mitochondria to persist, generating increased tumor-promoting free oxygen radicals reviewed in ref. 49).

Cytochrome oxidase subunit 2 is a key enzyme of the respiratory chain, catalyzing electron transfer from NADH and succinate to molecular oxygen (51). It has no direct tumor related function but serves as a marker for the cellular mitochondria content. Increased mitochondria content in cancer cells often occurs as a result of the elevated metabolism and energy needs of expanding tumor cell populations (52). Although the mitochondrial content provided no additional prognostic information in multivariate analysis, the marked prognostic relevance of MTCO2 immunostaining found in this study may still suggest ‘mitochondria content’ as a biomarker with potential clinical utility. Molecular analyses are frequently done in breast cancer to better assess patient prognosis and to determine whether adjuvant chemotherapy should be applied (6-8). Most currently used tests are analyzing RNAs of multiple genes forming a prognostic score (9-11,53). RNA based tests share the disadvantage, however, that the analyzed RNA always represents a mixture of cancer cells and a variable fraction of non-neoplastic inflammatory and stromal cells. Now that multiplex fluorescent-based quantitative IHC becomes increasingly available, it is well possible that RNA based test will sooner or later be replaced by IHC based multi-gene tests. MTCO2 might be a candidate for being part of such a test, also because of the general biologic importance of mitochondria, which are also the target of several anti-cancer drugs under development reviewed in refs. 54-57).

It is a limitation of our study that MTCO2 IHC data highlight relevant associations between cancer phenotype and genotype but do not provide mechanistic insights into the putative cancer biological role of MTCO2. Further studies on the tumor relevant aspects of mitochondrial density and MTCO2 protein function are required to better understand the prognostic role of MTCO2 in breast cancer.

In summary, our findings identify MTCO2 immunostaining as a powerful prognostic biomarker in breast cancer. MTCO2 measurement, most likely in combination with other antibodies might be of clinical utility in breast cancer prognosis assessment.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors’ contributions

All authors contributed to the conception and design of the study. PL, KS, MK, IW, PP, LT, CW, UM, BS, IvL, TK, RHK and FJ prepared the material, and collected and analyzed the data. PL, EB, RS, MK and GS wrote the first draft of the manuscript, and all authors commented on previous versions of the manuscript. RS, MK and GS confirmed the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The usage of archived diagnostic leftover tissues for manufacturing the tissue microarrays and their analysis for research purposes, as well as patient data analysis, has been approved by local laws (HmbKHG, §12) and by the local ethics committee (Ethics Commission of the Ärztekammer Hamburg, Hamburg, Germany; approval no. WF-049/09). Informed consent was waived by the ethics committee due to the retrospective nature of the study. All work has been carried out in compliance with the Declaration of Helsinki.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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