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TITLE: Microtubule-Associated Protein Expression and Predicting Taxane Response

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The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.
**We hypothesized that in addition to its predictive value, the microtubule-associated marker tau (MAP-tau) may also function as a prognostic biomarker.** The dual functionality of MAP-tau may translate into increased tumor molecular screening information for patients with breast cancer resulting in better treatment options. The results of this work indicate that MAP-tau functions as a prognostic marker for paclitaxel sensitivity when examined using automated quantitative analysis (AQUA) and tissue arrays YTMA 49-5 and YTMA 49-6. Each array contained approximately 750 tumor histospots. This work demonstrates that MAP-tau may be useful for further differentiating ER (+) and ER (-) patients and that increased MAP-tau expression in newly diagnosed breast cancer patients is associated with better outcome. Our findings suggest that MAP-tau may be a useful prognostic marker in addition to its predictive value for taxane response.
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Introduction

Breast cancer is the leading cause of cancer death in women between the ages of 20 and 59 accounts for more than 31% of all new cancers diagnosed in women and is the leading cause of death for women worldwide [1, 2]. While breast cancer family history is an important risk factor, sporadic cases account for more than 90% of all breast cancers and the etiology of this cancer remains largely unknown [3]. Clinical treatment, such as chemotherapy, currently relies on physical examination, imaging, histopathological information, tumor size, lymph node status, degree of metastasis, and biomarker expression (ER, PR, HER2) [4].

Microtubule stabilizing proteins, such as tau, have begun to gain attention as predictive markers. Tau expression has been found to decrease microtubule vulnerability to taxanes such as paclitaxel and its expression makes cells resistant to taxane treatment. Similarly it has recently been shown that low Tau is predictive for response to paclitaxel in breast cancer [5].

Current breast cancer therapy involves the use of taxanes such as paclitaxel and docetaxel [6]. Low tau expression has been shown to be predictive for response to paclitaxel. However, the prognostic value of tau has not been established [5]. This study examined MAP-tau expression in relation to overall patient survival at five years.

Body

In Aim 1 of this project, MAP-tau expression was measured in a large retrospective breast cancer cohort (n=480) with 20 year follow-up using tissue microarray technology and automated quantitative analysis (AQUA). The AQUA system used cytokeratin to define pixels as breast cancer within the array spot, and measured the intensity of tau expression using Cy5 conjugated antibodies. AQUA scores were correlated with clinical and pathologic variables.

MAP-tau showed a normal distribution of expression with high correlation (R= 0.76) between redundant cores. Kaplan-Meier survival analysis with a validated optimal cut-point showed a five year survival rate of 82% for high expressors versus only a 60% survival rate for low expressors (log rank, P<.0001). High tau expression correlated strongly with negative lymph node status (P = 0.0007). Univariate analysis indicated a protective relationship between tau expression and outcome (OR = 0.625, 95% confidence interval [CI] = 0.52-0.75; P<.008).
Task 1: Confirmation of tau as a predictive marker for paclitaxel sensitivity using AQUA

Tau as a predictive marker for paclitaxel sensitivity was confirmed using tissue whole sections and arrays and quantitative analysis was conducted with AQUA.

The following items from the Statement of Work have been **completed**:

a. Order breast test arrays and conduct antibody titration of tau using breast test arrays.
   **Completed**: Tau antibody was titrated using US Biological T1029 mouse monoclonal antibody. Optimal titration: 1:750.

b. Tau antibody staining of the Tissue arrays.
   **Completed**: YTMA 49-5 and YTMA 49-6 were stained with tau T1029 antibody.

c. Image collection, AQUA analysis, and image validation (Appendix A).
   **Completed**. (Fig. 1)

d. AQUA score statistical analysis
   **Completed** (Fig. 2 and Fig. 3; Tables 1, 2, and 3).

Timeline: Months 1-2
Figure 1. MAP-tau expression was studied in a large retrospective breast cancer cohort (n=656) with long term follow-up using tissue microarray at two-fold redundancy. Automated quantitative analysis (AQUA™) was used for in-situ analysis of protein expression. DAPI was used to define the nuclear region throughout the histospot (A). Cytokeratin was used to define pixels as breast cancer (tumor mask) versus stroma within the histospot (B), and Tau expression was measured using a Cy5-based detection system in the cytoplasm and the nuclear compartments within the tumor mask previously defined by the cytokertin (C). Analysis by AQUA showed a high correlation between cytoplasmic and nuclear tau, so total tau under the mask was used for analysis. AQUA scores for #347: 125.6, 126.7, 136.8 for total tau in tumor mask (shown in C), tau in nuclei and tau in cytoplasmic compartments, respectively (not shown).

Figure 2. Linear Regression of Microarray YTMA 49-4 and YTMA 49-5 AQUA Scores.
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<td>Median follow-up time</td>
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<td>Median age of diagnosis</td>
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<td>Censored (20 years)</td>
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<td>Uncensored (20 years)</td>
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<td>~27% tamoxifen (post 1978)</td>
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<td>Node Negative Treatment:</td>
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<td>Local Radiation and surgical resection only</td>
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Table 2. Cox Univariate Analysis

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<td>Tau</td>
<td>0.625 (0.51-0.75)</td>
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Table 3. Multivariate analysis of tau and histopathologic variables of breast cancer (5-year survival, n=364)

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<td>Nodal Status</td>
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<td>Total Nodes</td>
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<td>Estrogen Receptor (ER)</td>
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<td>Progesterone Receptor (PR)</td>
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<td>Tumor Size</td>
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<tr>
<td>Tau</td>
<td>0.732 (0.57-0.93)</td>
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Figure 3. Kaplan-Meier survival analysis for MAP-tau stratified by ER (-) status (panel A) and ER (+) status (panel B).
**Task 2: Construction of a Taxane Therapy tissue microarray as a training cohort for future predictive markers beyond tau.**

A retrospective cohort of patients treated with taxane therapy will be assembled and tissue samples from this cohort will be used to examine tissue heterogeneity.

The following items from the Statement of Work have been **completed:**

a. Select primary breast carcinoma tumors from the Yale Pathology archives or clinical trials that underwent taxane therapy.  
**Completed:** 140 whole tissue sections were obtained (Appendix B).

b. Design cell line controls for microtubule stabilizing proteins  
**Completed:** 6 YTMA 94-1 microarrays were stained to provide controls for whole tissue sections (Appendix C).

c. Analysis of whole tissue sections and tissue microarrays to examine tissue heterogeneity.  
**In Progress.** 39 of the 140 whole tissue sections have been analyzed (Appendix D and E). Problems with some tissue loss due to whole sections being floated on the slides rather than previous use of tape-transfer method.

**Timeline:** Months 3-12

**Key Research Accomplishments**

- This work demonstrated that Tau functions as a prognostic marker for paclitaxel sensitivity using AQUA and the Tissue Arrays YTMA 49-5 and YTMA 49-6.

- MAP-tau may be useful for further differentiating ER (+) and ER (-) patients

- Increased MAP-tau expression is associated with better outcome in breast cancer patients.

- MAP-tau may be a useful prognostic marker in addition to its predictive value for taxane response.

- Examining tissue heterogeneity using both whole tissue sections and tissue microarrays can provide important information regarding the usefulness of tissue microarrays in cancer diagnosis and treatment.
Reportable Outcomes

1. San Antonio Breast Cancer Symposium abstract acceptance and poster presentation
2. YTMA 49-4 and YTMA 49-5 tissue microarrays stained with T1029 MAP-tau Mab
   (Appendix A and Fig. 1).
3. Whole Section Tissue database with 15, 604 images. (Appendix E and F)
4. 6 control slides created: YTMA 941 tissue microarray with 120 histospots
   (Appendix E and F).
5. Data Characterization Algorithm for coding tumor tissue (Appendix E)
6. PhD dissertation research project that is specifically and uniquely breast cancer-focused
   in Department of Experimental Pathology program at Yale University with
   mentoring and training emphasis in breast cancer research that would not be possible
   without this grant.

Conclusion

The current research findings indicate that increased MAP-tau expression is associated
with better outcome, that MAP-tau may be useful for further differentiating ER (+) and
ER (-) patients, and that MAP-tau may serve as a prognostic marker in addition to its
predictive capabilities. The next phase of this project will examine additional microtubule
related proteins to compare with MAP-tau.

Our findings may be reflective of increased mitotic arrest and inhibition of cellular
proliferation within cancer cells that can occur when high levels of MAP-tau are present.
Taxanes function in a similar manner to MAPs by binding and stabilizing microtubules
leading to mitotic arrest in cancer cells. Thus, taxanes may be competing for binding
sites with tau and this may explain why increased MAP-tau expression results in
resistance to taxane treatment (lack of functional binding sites available for paclitaxel)
and why low MAP-tau expression is predictive for paclitaxel response (abundance of
functional binding sites available for paclitaxel).

The dual functionality of MAP-tau may translate into increased tumor molecular
screening information for patients with breast cancer resulting in better treatment options.
Consequently, other microtubule associated proteins may also serve as valuable
biomarkers for the personalized molecular assessment of breast cancer tumors and we are
working to systematically evaluate these proteins.
References

Appendices
Appendix A

Automated Quantitative Analysis (AQUA)

What is an AQUA Score?

- Each pixel within the mask is assigned a user-defined subcellular compartment (or unassigned)
- The intensity of the “target” of interest is measured on a scale of 0-255 in each pixel in each compartment
- The final score is normalized by dividing the total target intensity by the area of each subcellular compartment.
- The final score is proportional to a number of molecules per unit area.

Methods and Instruments
**AQUA Analysis of Tissue**

The AQUA software linked to the fluorescence microscopy system allows for quantification of the protein of interest within the tumor region of each tissue microarray core.

Step 1: Cytokeratin is used to separate epithelial tumor from surrounding stroma, creating a tumor mask.

Step 2: Different fluorescent tags (like DAPI, Cy-5 tyramide) are used to demarcate subcellular compartments (nuclear, membrane, cytoplasmic, etc).

Step 3: Due to the thickness of the tissue sections and the resulting overlap of compartments, a rapid exponential subtraction algorithm (RESA) is used to subtract an out-of-focus image from an in-focus image, providing improved pixel assignment to subcellular compartments. An AQUA score is generated for each compartment ranging from 0-255 (see box *What is an AQUA score...*)

Step 4: At the Cy-5 wavelength, which is outside the range of tissue autofluorescence, the target of interest is tagged and measured within the subcellular compartments by the PLACE algorithm.

The resulting AQUA score is the measurement of the biomarker pixel intensity within a compartment divided by the total area of the compartment (to normalize for differences in tumor area in each spot).
Appendix B

TAX 307 Whole Tissue Sections
Patient Characteristics and Study Design

• Cases obtained from the TAX 306 Study Group (2003) (Dr. Lyndsay Harris, Yale Breast Cancer Center)

• Study Design:
  - Multicenter: 58 total in Europe, S. Africa, S. America
  - Randomized (centralized)
  - Non-blinded
  - Phase III

• Objective: compare efficacy & safety AT vs AC as 1st line chemotherapy in 429 patients w/ untreated MBC
  - AT: doxorubicin (DNA intercalation & anthracycline) + docetaxel
  - AC: doxorubicin and cyclophosphamide (alkylating agent)

• Treatment regimen: AT or AC on day 1, every 3 weeks for 8 cycles

• Primary endpoints: Time to treatment progression (TTP)

• Secondary endpoints: overall response rate (ORR), time to treatment failure (TTF), toxicity, survival, quality of life (QoL)

• Inclusion criteria:
  - adjuvant or neoadjuvant non-anthracycline chemo OK
  - prior hormonal therapy OK, but not concurrent
  - NO previous taxanes

• TAX 307 cohort: 140 cases from AT arm
Appendix C

TAX 307 Whole Tissue Sections
Methods

• 140 cases:
  - Floated, whole tumor sections
  - PLUS slides inconsistently used

• 85 matching H&E slides

• 6 control slides: YTMA 94-1; Cell lines for secondary normalization + staining quality control

• Staining:
  – 6 consecutive batches:
  – 25 slides/batch + 1 YTMA 94-1
  – 1 week period: early November 2006

• Target:
  - MAP-tau mouse monocolonal antibody
  - US Biological; 1:750 dilution (titrated)

• Image Capture:
  - HistoRx Image Grabber

• Quantitative analysis of specimens:
  - HistoRx AQUA
Appendix D

29th Annual San Antonio Breast Cancer Symposium

Abstract Number: 551023

Contact/Presenting Author: Maria T. Baquero

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Address: 310 Cedar Street/ BML 163, PO Box 208023
City/State/Zip/Country: New Haven, CT, 06520-8023, United States
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Abstract Categories: 11. Prognostic Factors

Disclosure: There is no financial interest/arrangement or affiliation with one or more organizations.
Off Label: No

Title: Microtubule-associated protein (MAP)-tau is a prognostic biomarker associated with better outcome in breast cancer.

Maria T. Baquero, MPH1, Mark Gustavson, PhD1, Jena Giltnane, MS1, Robert L. Camp, MD, PhD1 and David L. Rimm, MD, PhD1. 1Department of Pathology, Yale School of Medicine, New Haven, CT, 06520.

Body: Background: Clinical treatment involving adjuvant or neoadjuvant treatment currently relies on a variety of factors such as tumor size, lymph node status, degree of metastasis, and biomarker expression (ER, PR, HER2). However, additional markers that can identify subsets of patients requiring more aggressive or pathway-targeted adjuvant treatments are needed. Microtubule-associated proteins, such as tau, have recently begun to gain attention as both predictive and prognostic markers. These proteins promote the assembly of tubulin monomers into microtubules functioning to stabilize microtubules and thus working against cancer by inducing mitotic arrest. Tau expression has been found to decrease microtubule vulnerability to taxanes and its expression makes cells resistant to taxane treatment. In addition, low tau expression has been shown to be predictive for response to the taxane, paclitaxel, in breast cancer. However the prognostic value of tau has not been established.

Material and Methods: Tau expression was measured in a large retrospective breast cancer cohort (n=480) with 20 year follow-up using tissue microarray technology and automated quantitative analysis (AQUA). The AQUA system used cytokeratin to define pixels as breast cancer within the array spot, and measured the intensity of tau expression using Cy5 conjugated antibodies. AQUA scores were correlated with clinical and pathologic variables.

Results: Tau showed a normal distribution of expression with high correlation (R= 0.76) between redundant cores. Kaplan-Meier survival analysis with a validated optimal cut-point showed a five year survival rate of 82% for high expressors versus only a 60% survival rate for low expressors (log rank, P<.0001). High tau expression correlated strongly with negative lymph node status (P = 0.0007). Univariate analysis indicated a protective relationship between tau expression and outcome (OR = 0.625, 95% confidence interval [CI] = 0.52-0.75; P<.0001)

Discussion: Similar to microtubule-associated proteins such as tau, taxanes also bind and stabilize microtubules leading to mitotic arrest in cancer cells. Thus, taxanes may compete for binding sites with tau and this may explain why increased tau expression results in resistance to taxane treatment (lack of functional binding sites available for paclitaxel) and why low tau expression is predictive for paclitaxel response (abundance of functional binding sites available for paclitaxel). This study found that increased tau expression is associated with better outcome. This may be reflective of increased mitotic arrest and inhibition of cellular proliferation within cancer cells that can occur when high levels of tau are present. The biological basis of high tau expression and breast cancer pathogenesis requires further investigation. These findings suggest that tau may be a useful prognostic marker in addition to its predictive value in taxane response.
- Spindle formation
- Chromosome segregation
- Railroad tracks of the cell

Kumar M.R. Bhat and Vijayasaradhi Setaluri (2007), AACR
TAX 307
Worksheet
Of Images Collected

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**TOTAL**

|     | 1113 | 918  | 1627 | 1573 |
### TAX 307 Image Worksheet

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| TOTAL IMAGES | 15604 |
Image Capture: HistoRx Image Grabber
Saturation Function ↓
Image Grabber: AQUA Score Normalization through Saturation Function

\[ P_i = \frac{P(saturation)}{Exp\ time} \]

\[ \text{Power} = \frac{\text{Intensity}}{\text{Time}} = P_i \]

- Each pixel: 1-256
- Calculate avg total pixel saturation
- Black --> White
- Unsaturated --> Saturated

\[ P_{\text{SAT}}/ 4,194,304 \text{ Total Pixels} \times 100 = \% \text{ saturation} \]
Image Capture: Data Set Descriptors

- Project Parameters
  - 184.4 GB total
  - 149 folders (1 folder/case)
  - 845 files

Typical Case Slide Size Range:

**Low:** Case # H77
- 90 MB
- 1 ROI/TMA
- 10 images total

**High:** Case # H99
- 6.94 GB
- 8 ROI/TMAs
- 519 images total
Image Capture Challenges

- Only 61% of H&Es available
- Tissue distortion after staining: folding, tearing, shearing, erosion
- Sections not planar; Multiple ROIs
- First use 20x to “explore” tissue sections and plane of focus, then set ROIs. Collapse any ROI into 2 if still out of focus
- PM3 makes many, many files! Label appropriately for patient cross-matching late

- Criteria for Excluding Cases (determined at beginning of study):
  1. No tissue on specimen slide
  2. Heavy tissue erosion: >95% of tissue gone
  3. Validation: out of focus, artifacts (uncropable)

Total Cases Excluded during image capture: 16
(16/140 = 11.4% Excluded, 88.6% Retained)
Quantitative analysis of specimens: HistoRx AQUA
Quantitative analysis of specimens: HistoRx AQUA

Strengths:

• Rapid experiment run-time: ~11 TMA images per minute
• Tumor Histogram Threshold: 10-20% and can be adjusted per slide
• Relatively small template and analysis file size: <100 MB
• User-friendly interface with multiple ways to examine images

Limitations:

• No Cropping! so entire image must be discarded
• Still some bugs so error messages are common with too many open windows
• Exposure times may not register properly in manual mode
H1
H1
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Data Analysis

• 15 histograms from beginning, middle, end of cohort for AQUA score distribution

• Coding of all cases:
  • Invasive
  • Mixed DCIS + Invasive
  • No tumor, normal ducts present
  • Stroma, no ducts, no tumor
  • Blank, no tissue
  • Technical artifact (blurry, lint, etc)
  • Other
  • Show Pathologist
Case: H2.1
AQUA
Score: 40.68

Range: 25-50

Tumor Mask

Target: MAP-tau
Case: H5.2

AQUA Score: 63.77

* Range: 50-100
Case: H5.3

AQUA
Score: 1020.77
Case: H5.4

AQUA

Score: 2012.27

* Range: 2000-2050

Tumor Mask

Target: MAP-tau
Case: H23.5

AQUA

Score: 2239.94

*Range: 2000-2250
Case: H34.6

AQUA
Score: 277.46

*Range: 250-375
Case: H34.7

AQUA

Score: 1997.81

Range: 1875-2000
Case: H34.8
AQUA Score: 3376.37

Tumor Mask

Target: MAP-tau
Case: H82.9

AQUA
Score: 105.16

* Range: 100-150
Case: H82.10

AQUA
Score: 587.53

Tumor Mask

Target: MAP-tau
Case: H125.11

AQUA
Score: 106.12

Tumor Mask

Target: MAP-tau
Case: H125.12

AQUA

Score: 973.06

Tumor Mask: % Target: MAP-tau

* Range: 900-1000
Case: H138.13

AQUA
Score: 173.79

Range: 150-200

Tumor Mask

Target: MAP-tau
Case: H138.14

AQUA

Score: 426.82

Range: 400-450

Quantiles and Moments:

- 100.0% maximum: 688.57
- 99.5% maximum: 688.57
- 97.5% maximum: 636.17
- 90.0% maximum: 502.42
- 75.0% quartile: 390.26
- 50.0% median: 230.95
- 25.0% quartile: 149.36
- 10.0%: 86.59
- 2.5%: 39.98
- 0.5%: 39.36
- 0.0% minimum: 39.36

* Range: 400-450
Case: H138.16

AQUA
Score: 619.50

* Range: 600-650
TAX307 Cell Line AQUA Score Regression for YTMA 94-1 Arrays

\[ y = 1.0084x + 324.68 \]

\[ R^2 = 0.7148 \]

\[ R = 0.8454 \]
TAX307 Cell Line Distribution Grouped

![Graph showing AQUA Score for MB468, BT474, ZR75.1, T47D, and MB231.](image)

- MB468
- BT474
- ZR75.1
- T47D
- MB231
y = 1.0084x + 324.68
$R^2 = 0.7148$
R = 0.8454

TAX307 Cell Line AQUA Score Regression for YTMA 94-1 Arrays