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# Correlative Study of Tumor Hypoxia and Metastatic Potential in Breast Cancer

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ABSTRACT

Background: Human tumor hypoxia is associated with poor prognosis independent of treatment modality. In addition to radioresistance, hypoxia induced stress factors may lead to malignant progression.

Purpose: Using a novel physiological approach to detect tumor hypoxia with pimonidazole in human breast tumors, the aims are to study relationships between hypoxia and biomolecular markers of cell proliferation (PCNA), angiogenesis (MVD-microvessel density, VEGF), p53, apoptosis, and regional nodes as clinical indicator of metastases.

Methods: Breast cancer patients enrolled in an IRB approved study receive pimonidazole intravenous infusion. Breast tumor biopsy specimens are examined with immunohistochemical techniques for pimonidazole binding (hypoxia) and for the above biomolecular markers. Regional node metastases data are recorded.

Results: Twelve patients have been enrolled on the study. Tumor hypoxia detected with pimonidazole binding ranges from 0-33 % in the tumor biopsies examined to date. MVD ranges from 2-82 vessels per field. Other studies are awaiting additional patient enrollment. There are no adverse reactions to the pimonidazole infusion (Appendix I-III).

Conclusions: This is the first demonstration of tumor hypoxia detection in human breast cancer using pimonidazole. These data suggest it will be a valuable technique for correlative studies of tumor hypoxia with both clinical and biomolecular markers of tumor aggressiveness.

Scope: The specific aims are:

I: Determine the presence and extent of tumor hypoxia in biopsies of primary breast cancer using pimonidazole binding to hypoxic tumor cells.

II: Determine the patterns of pimonidazole binding in the breast cancer biopsies in relation to other landmarks such as blood vessels and necrosis.

III: Correlate the presence and extent of tumor hypoxia in primary breast cancer with the presence of axillary node metastases.

IV: Correlate the presence and extent of tumor hypoxia with the presence of other biological markers: p53, apoptosis, PCNA, and VEGF.

V: Monitor adverse effects of pimonidazole.
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M. Mandarino  
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Jan 4, 2007  
Date
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INTRODUCTION

Background: Tumor hypoxia predicts for poor prognoses in human tumors independently of whether chemotherapy, radiotherapy or surgery are used as treatments. The hypoxic tumor microenvironment may be a significant factor in cellular processes involved in tumor proliferation and metastases. It has been postulated that this may be due to the induction of oxygen regulated proteins such as vascular endothelial growth factor (VEGF).

Purpose: A novel physiological approach using pimonidazole for tumor hypoxia assessment in human breast tumors has been initiated to study the metastatic potential of primary breast cancer cells. Does tumor hypoxia induced oxygen stress proteins such as vascular endothelial growth factor (VEGF) lead to tumor aggressiveness through cell proliferation and metastases. Does the presence of hypoxia in the primary breast tumors detected by pimonidazole immunohistochemical binding correlate with the presence of axillary lymph node metastases and the presence of markers of cell proliferation, p53, apoptosis, and VEGF in the primary breast tumor tissue.

Scope: The specific aims are:

I: Determine the presence and extent of tumor hypoxia in biopsies of primary breast cancer using pimonidazole binding to hypoxic tumor cells.

II: Determine the patterns of pimonidazole binding in the breast cancer biopsies in relation to other landmarks such as blood vessels and necrosis.

III: Correlate the presence and extent of tumor hypoxia in primary breast cancer with the presence of axillary node metastases.

IV: Correlate the presence and extent of tumor hypoxia with the presence of other biological markers: p53, apoptosis, PCNA, and VEGF.

V: Monitor adverse effects of pimonidazole.

Research Progress Associated with Tasks outlined in the Statement of Work.

Task 1: Patient enrolment

After the initial award (IRB) of the grant, a number of changes in the Consent Form approved by our Institutional Review Board were requested by the Surgeon General's Human Subjects Research Review Board (HSRRB, HURRAD Log. No. A-7766), USAMRMC Human Subjects Protection Division. Although most of these changes could be addressed without significant difficulty, a major problem related to the provision of financial compensation for research subjects in the event of research related injury. The required language of the two Review Boards were in direct conflict.

Following further discussions on this subject, language acceptable to both Review Boards was developed whereby Department of Defense as the sponsor of the research assumes the financial responsibility. These deliberations and required approval of the Consent Form has delayed entry of research subjects entry into the research protocol for this grant. There was a significant time lapse in obtaining agreement and approvals of the Informed Consent Form from the Institutional Review Boards for participation by breast cancer patients in the research study funded by this grant. As a result patient enrolment was delayed and slow but is now actively progressing.
For each patient enrolled:

**Task 1A**
- a. Document eligibility criteria upon enrollment.  
  **Done.**
- b. Have signed informed consent on record prior to study procedures.  
  **Done**
- c. Record clinical data.  
  **Done**

**Task 1B**
- a. Administer pimonidazole, 0.5 g/m² in 100 ml N-saline i.v.  
  **Done**
- b. Procure breast biopsy specimen at the time of lumpectomy/mastectomy.  
  **Done**
- c. Prepare biopsy material for pimonidazole and biological markers staining.  
  **Done**
- d. Record pathological status of axillary lymph nodes.  
  **In progress**

**Task 1C**
- a. Perform pimonidazole immunostaining.  
  **In progress**
- b. Perform p53, apoptosis, PCNA, Ki-67, and VEGF marker staining  
  **Awaiting batch study**
- c. Record any adverse effects of pimonidazole  
  **Done**

**Task 1D**
- a. Perform Image Analysis on stained slides.  
  **In progress**
- b. Calculate Hypoxic Fraction (ratio of labeled to unlabeled cells).  
  **In progress**
- c. Record p53, apoptosis, PCNA, Ki-67, and VEGF marker staining.  
  **Awaiting batch study**
- b. Assess hypoxia marker binding relationships to anatomic landmarks.  
  **Awaiting batch study**

**Task 2**  **Perform Data and Statistical Analysis to obtain results of Specific Aims I-V**

**See Preliminary Data Below**

**Preliminary Data and Results**

Pimonidazole labeling of breast cancer cells has been demonstrated in the tumor biopsy specimens of the patients enrolled in this DOD supported research study. These preliminary results were combined with the results from breast cancer patients enrolled in our own IRB approved study and were recently presented at the 22nd Annual San Antonio Breast Cancer Symposium. Copy of the abstract is attached in Appendix I.

Detection and estimation of the presence of tumor hypoxia in the primary human breast cancers show a range of 0-33% in the tumor biopsy analysis to date. MVD ranges from 2-82 vessels per field. Overall high MVD correlated with low hypoxia (p=.001). That is hypoxia does not influence MVD. Rather, tumor vascularity determines the extent of hypoxia in human breast adenocarcinomas. These preliminary data suggest that the generation of high MVD is not the mechanism by which hypoxia leads to poor prognoses measured at the time of clinical presentation. This does not rule out the possibility that hypoxia plays a role in angiogenesis at earlier stages in the natural history of tumors.

These results have been also been accepted for presentation at the American Radium Society Meeting, March, 2000, London, United Kingdom, (Appendix II), and at the Radiation Research Society Meeting, Albaquerque, New Mexico, April, 2000 (Appendix III).

No adverse side effects have been observed from the pimonidazole infusion to the patients entered on this study.
KEY RESEARCH ACCOMPLISHMENTS AND REPORTABLE OUTCOMES:

The study is ongoing and results will be updated as more breast cancer patients are enrolled on the study. However, we note with enthusiasm the following significant observations from the preliminary results:

1. An innovative method of tumor hypoxia detection has been demonstrated using pimonidazole. This is the first demonstration of hypoxia detection using this immunohistochemical technique in human breast cancers. In addition, image analysis quantification of the extent of hypoxia has been accomplished.

2. Innovative correlative study of tumor hypoxia detection with microvessel density analysis has been performed using a double staining technique on the tumor section on the same slide.

3. Abstracts are provided in Appendix I, II, and III.

4. Dr. Ballenger has been awarded a Travel Fellowship by the Radiation Research Society to present the results from this research at their April, 2000 meeting.

5. Dr. Ballenger’s research work on this project has been accepted as the research requirement for her Radiation Oncology training in the University of North Carolina Hospitals Residency Program.

CONCLUSIONS:

This is the first report of tumor hypoxia detection using pimonidazole immunohistochemical method in human breast cancers (Appendix I). Results from this breast cancer research will be updated as more patients are entered on the study and further analysis of the tumor biopsies in progress are completed. Dr. Ballenger has been provided with research training and her contributions have led to 3 abstracts and a Travel Award Fellowship of the Radiation Research Society. On account of the patient accrual difficulties as noted above, an extension to November 1st, 2001 is requested. This will allow accrual of additional patients on IRB approved human research protocol and subsequent laboratory and data analysis.

REFERENCES:  NONE
APPENDICES

Attach all appendices that contain information that supplements, clarifies or supports the text. Examples of appendices include journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.


Appendix III. Hypoxia and microvessel density in human breast adenocarcinomas. 1Ballenger CA, 1Varia MA, 1Chou S-C, 2Novotny DB, 3Haroon Z and 1Raleigh JA. Radiation Research Society Meeting, Albaquerque, New Mexico, April 29-May 3, 2000.
APPENDIX I

Hypoxia And Microvessel Density In Human Breast Adenocarcinomas.

1Ballenger CA, 1Varia MA, 1Chou S-C, 2Novotny DB, 3Haroon Z and 1Raleigh JA.

1Radiation Oncology and 2Pathology, UNC School of Medicine, Chapel Hill, NC 27599; 3Radiation Oncology, Duke University Medical Center, Durham, NC 27710.


Tumor hypoxia predicts for poor prognoses in human tumors independently of whether chemotherapy, radiotherapy or surgery are used as treatments. It has been postulated that this is due to the induction of oxygen regulated proteins such as vascular endothelial growth factor (VEGF). However, VEGF is not expressed in the majority of hypoxic cells in human tumors so an examination of a product of VEGF action, microvascularization, has been examined as a possible link between hypoxia and poor prognosis. Microvascular density (MVD) has been measured in 16 patients with breast adenocarcinomas. Immunostaining for pimonidazole adducts was used to detect hypoxia and immunostaining for Factor VIII or transglutaminase were used to detect microvasculature. In the case of MVD, 5 fields (0.196 um²) of highest MVD in a section from each of 4 biopsies per tumor were selected and scored. Isolated clusters of endothelial cells, single endothelial cells and branching structures were counted as one vessel. Factor VIII and transglutaminase gave similar results and interobserver agreement was excellent. Considerable intrabiopsy and interbiopsy variation was observed. MVD ranged from 2 to 82 vessels per field. Overall, high MVD correlated with low hypoxia (p = 0.001). We conclude that hypoxia does not control microregional vascular density but, instead, tumor vascularity determines the extent of hypoxia in human breast adenocarcinomas. We further conclude that the generation of high, microregional MVD is not the mechanism by which hypoxia leads to poor prognoses measured at the time of clinical presentation. This does not rule out the possibility that hypoxia plays a role in angiogenesis at earlier stages in the natural history of breast tumors. Supported by DHHS R 42 CA68826 and DOD BC962506.
Appendix II.

**Hypoxia and microvessel density in human breast tumors.**

1Ballenger CA, 1Chou S-C, 1Calkins-Adams DP, 2Novotny, DB, 1Raleigh JA, 1Varia MA.

1Radiation Oncology and 2Pathology, UNC School of Medicine, Chapel Hill, NC 27599.

American Radium Society Meeting, April 1-5, 2000, London, United Kingdom.

**ABSTRACT:**

It has been reported that tumor hypoxia predicts for poor prognoses in human tumors independently of whether chemotherapy, radiotherapy or surgery are used to treat the tumors. Given the possibility that tumor hypoxia leads to poor prognosis by stimulating microvasculature, microregional hypoxia and microvascular density (MVD) has been measured in 16 patients with breast adenocarcinomas.

Immunostaining for pimonidazole adducts was used to measure hypoxia and immunostaining for Factor VIII was used to measure microvasculature. In the case of MVD, 5 fields (0.196 mm$^2$) of highest MVD in sections from 4 biopsies per tumor were selected and scored. Isolated clusters of endothelial cells, single endothelial cells and branching structures were counted as one vessel.

MVD ranged from 2 to 82 vessels per field. Considerable intrabiopsy and interbiopsy variation was observed. Overall, high MVD correlated with low hypoxia ($p = 0.001$). That is, hypoxia does not control microregional vascular density. Rather, tumor vascularity determines the extent of hypoxia in human breast adenocarcinomas.

We conclude that the generation of high, microregional MVD is not the mechanism by which hypoxia leads to poor prognoses measured at the time of clinical presentation. This does not rule out the possibility that hypoxia plays a role in angiogenesis at earlier stages in the natural history of tumors.
Appendix III.

Hypoxia And Microvessel Density In Human Breast Adenocarcinomas.

1Ballenger CA, 1Varia MA, 1Chou S-C, 2Novotny DB, 3Haroon Z and 1Raleigh JA.

1Radiation Oncology and 2Pathology, UNC School of Medicine, Chapel Hill, NC 27599; 3Radiation Oncology, Duke University Medical Center, Durham, NC 27710.

Radiation Research Society Meeting, Albuquerquc, New Mexico, April 29-May 3, 2000

ABSTRACT

Tumor hypoxia predicts for poor prognoses in human tumors independently of whether chemotherapy, radiotherapy or surgery are used as treatments. It has been postulated that this is due to the induction of oxygen regulated proteins such as vascular endothelial growth factor (VEGF). However, VEGF is not expressed in the majority of hypoxic cells in human tumors so an examination of a product of VEGF action, microvascularization, has been examined as a possible link between hypoxia and poor prognosis. Microvascular density (MVD) has been measured in 16 patients with breast adenocarcinomas. Immunostaining for pimonidazole adducts was used to detect hypoxia and immunostaining for Factor VIII or transglutaminase were used to detect microvasculature. In the case of MVD, 5 fields (0.196 um²) of highest MVD in a section from each of 4 biopsies per tumor were selected and scored. Isolated clusters of endothelial cells, single endothelial cells and branching structures were counted as one vessel. Factor VIII and transglutaminase gave similar results and interobserver agreement was excellent. Considerable intrabiopsy and interbiopsy variation was observed. MVD ranged from 2 to 82 vessels per field. Overall, high MVD correlated with low hypoxia (p = 0.001). We conclude that hypoxia does not control microregional vascular density but, instead, tumor vascularity determines the extent of hypoxia in human breast adenocarcinomas. We further conclude that the generation of high, microregional MVD is not the mechanism by which hypoxia leads to poor prognoses measured at the time of clinical presentation. This does not rule out the possibility that hypoxia plays a role in angiogenesis at earlier stages in the natural history of breast tumors. Supported by DHHS R 42 CA68826 and DOD BC962506.