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14. ABSTRACT We ran an outstanding DOD project (W81XWH-06-1-0625) about hypoxia in invasion and metastasis by a novel technology. At first, we have successfully constructed a hypoxia-responsive fluorescence protein animal model for mammary carcinoma studying by using a mammary carcinoma cell line with constitutively expressed enhanced red fluorescence protein (RFP) as a tumor marker and green fluorescence protein (GFP) as a reporter for hypoxia and HIF- activation. The second, we identified and confirmed that increased HIF-1 expression were associated with the activation of genes essential for cell invasion and metastasis. Additionally, we did a window surgery to determine the behavior of hypoxic and non-hypoxic mouse mammary cancer cells in mice. We have successfully identified tumor cells expressed HIF-GFP and RFP under in vivo window image. Finally, we demonstrated that hypoxia and activating HIF-1 downregulate the DNA mismatch repair proteins (mlh1 and/or msh2), a group of important proteins for maintaining genetic stability. We think that our research discoveries are a major advance in not only understanding the basic biology of cancer but in also developing new insights into the mechanisms of invasion which cause the highest levels of morbidity and mortality among breast cancer patients.					
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Introduction

Metastasis represents the major cause of mortality in breast cancer patients, and the precise mechanism of metastatic breast cancer remains unknown. A number of studies have suggested that hypoxia may play important role in tumor development, growth, angiogenesis, apoptosis, invasion and metastasis (1, 2). Recently, an *in vivo* invasion assay has been developed, which provides an opportunity to collect primary tumor cells that are actively in the process of invasion (3). Multiphoton based intravital imaging technology is a powerful tool to observe tumor cell invasion and intravasation directly in rat and transgenic mouse mammary tumors (4). The combination of the *in vivo* invasion assay and intravital imaging technology made it possible, for the first time to directly identify the key characteristics of invading tumor cells and tumor microenvironment. In this proposed project, we hypothesize that hypoxia supplies the microenvironment that causes the changes in tumor cell behavior seen as cell motility, and triggers the invasion of tumor cells in the primary breast tumor. We will test our hypothesis by following specific Aims: 1. To investigate if hypoxia stimulates cancer cell invasion. We will identify and confirm the alteration in the level of multiple hypoxia inducible factors in the invasive breast cancer cells collected by the *in vivo* invasion assay. 2. To investigate the behaviors of invasive tumor cells under hypoxia, *in vivo* using multiphoton based intravital imaging in whole animals.

Body

1: Construction of a dual fluorescence breast cancer animal model

Method: A mouse mammary carcinoma cell line, 4T1, with constitutively expressed enhanced cyan fluorescence protein (CFP) as a tumor marker and green fluorescence protein (GFP) as a reporter for hypoxia and HIF-1 activation is used to generate tumors orthotopically in 20 SCID mice (Month 0-3).

Result: In this period, we have successfully constructed a hypoxia-responsive fluorescence protein animal model for mammary carcinoma studying by using a mammary carcinoma cell line with constitutively expressed enhanced red fluorescence protein (RFP) as a tumor marker and green fluorescence protein (GFP) as a reporter for hypoxia and HIF activation (See Figure1).

2: Investigation of the behaviors of invasive tumor cells under hypoxia

Method: We investigate the behaviors of invasive tumor cells under hypoxia by an *in vivo* invasion assay and a multiphoton based intravital imaging technology in 20 mice (Month 4-6). An intravital-Imaging technology will be used to determine the behaviors of hypoxic (GFP and CFP positive) and non-hypoxic (CFP only) mouse mammary carcinoma cells within the primary tumor by multiphoton microscope. Tumor area (as indicated by CFP fluorescence), tumor hypoxia (as indicated by GFP fluorescence), and vasculature are observed daily using a Zeiss intravital multiphoton microscope. We will characterize tumor cell behavior for speed of migration and mode of motility under hypoxia using an intravital multiphoton microscope by a mouse dorsal skin-fold window chamber. The behaviors of hypoxic (GFP and CFP positive) and non-hypoxic (CFP only) mouse mammary carcinoma cells, 4T1 within the primary tumor determined by multiphoton microscope

Result: We identified and confirmed that increased HIF-1 expression were associated with the activation of genes essential for cell invasion and metastasis (See Figure 2).

3: Investigate the hypoxia and activating HIF-1 downregulate the DNA mismatch repair proteins (mlh1 and/or msh2) (Month 7-12)

Methods: We performed a parallel analysis to identify DNA mismatch repair function (DNA mismatch repair proteins) in the level of multiple hypoxia inducible factors from invasive tumor cells collected by in vivo invasion assay. We determined the expression of DNA mismatch repair proteins in the level of multiple hypoxia inducible factors from invasive tumor cells. Changes in the level of multiple hypoxia related factor (HIF-1) and DNA mismatch repair proteins (MLH1, MSH2 and MSH6) in the invasive cells were determined by immunohistochemistry, Real-time PCR and Western blot assays.

Result: We measured that DNA mismatch repair proteins: MLH1 and MSH2 are significantly decreased in tumor cells with hypoxia compared to the tumor cells with no-hypoxia by immunohistochemistry (Figure 3). In addition, our results showed that the transcript and translation levels of MLH1 and MSH2 genes are significantly decreased in tumor cells with hypoxia (90% cells concentration) compared with tumor cells without hypoxia (10% cells concentration) using real-time PCR and Western blot assays, (Figure 4). These results indicated that hypoxia and activating HIF-1 downregulate the DNA mismatch repair proteins (mlh1 and/or msh2), a group of important proteins for maintaining genetic stability.

We think that our research discoveries are a major advance in not only understanding the basic biology of cancer but in also developing new insights into the mechanisms of invasion which cause the highest levels of morbidity and mortality among breast cancer patients.

Key research accomplishments

1. A hypoxia-responsive fluorescence protein animal model for mammary carcinoma studying has been successfully constructed by using a mammary carcinoma cell line with constitutively expressed enhanced red fluorescence protein (RFP) as a tumor marker and green fluorescence protein (GFP) as a reporter for hypoxia and HIF- activation.
2. We identified and confirmed that increased HIF-1 expression were associated with the activation of genes essential for cell invasion and metastasis.
3. A particularly interesting story was that hypoxia and activating HIF-1 downregulate the DNA mismatch repair proteins (mlh1 and/or msh2), a group of important proteins for maintaining genetic stability. Overall, there is considerable evidence now that hypoxia-perhaps largely through Hypoxia-HIF-MMR system is a potent promoter of genetic instability and metastasis.
4. We did a window surgery to determine the behavior of hypoxic and non-hypoxic mouse mammary cancer cells in mice.
5. We have successfully identified tumor cells expressed HIF-GFP and RFP under in vivo window image.

Reportable Outcomes

We think that our research discoveries are a major advance in not only understanding the basic biology of cancer but in also developing new insights into the mechanisms of invasion which cause the highest levels of morbidity and mortality among breast cancer patients.

Conclusions

We conclude that hypoxia promote tumor cells invasion and metastasis. We will submit some abstracts to AACR and ASHG meetings. In addition, we will further apply some grants to expend these studies in the near future.

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Appendices:

Figure 1

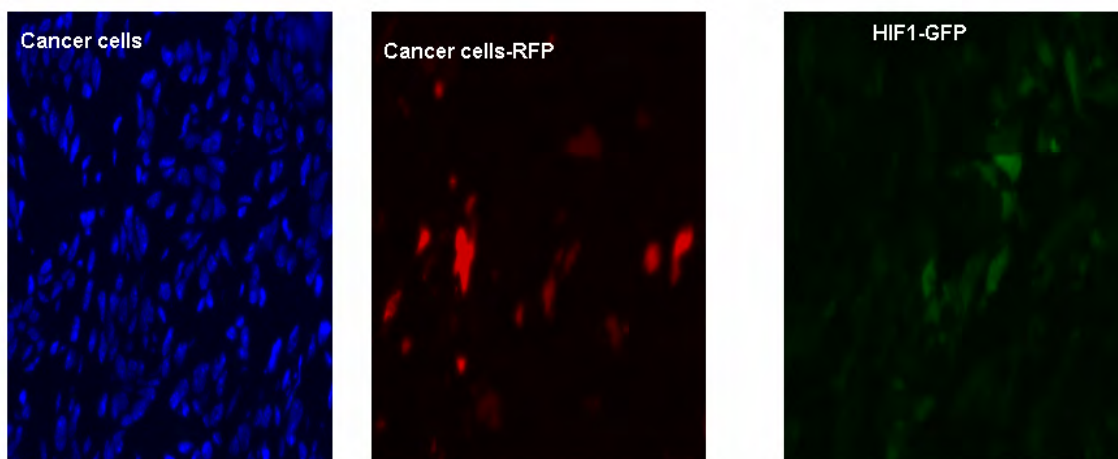


Figure 2: Hypoxia (HIF-1 expression) promoter cell invasion and metastasis

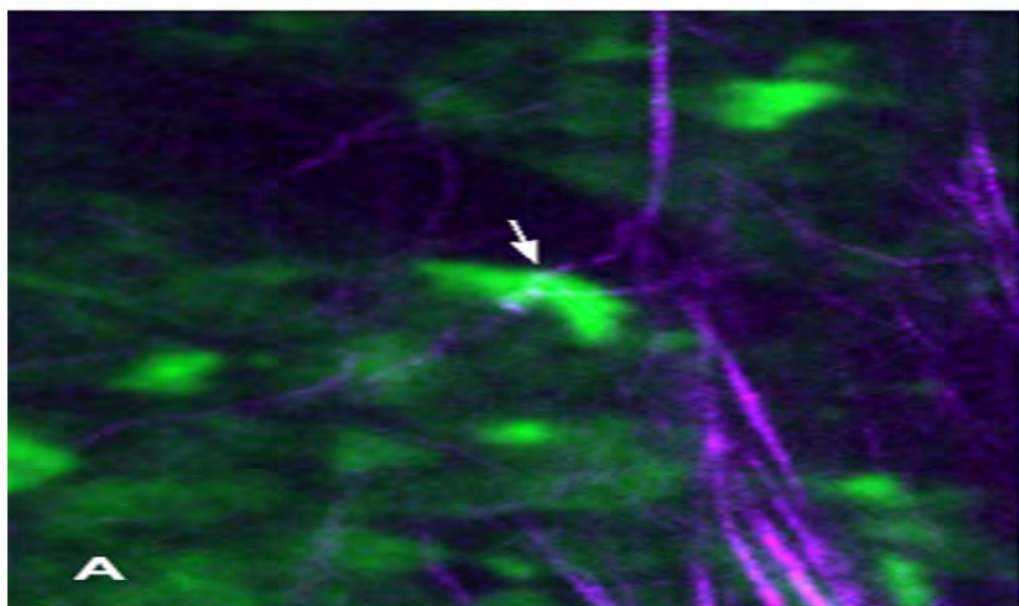


Figure 3

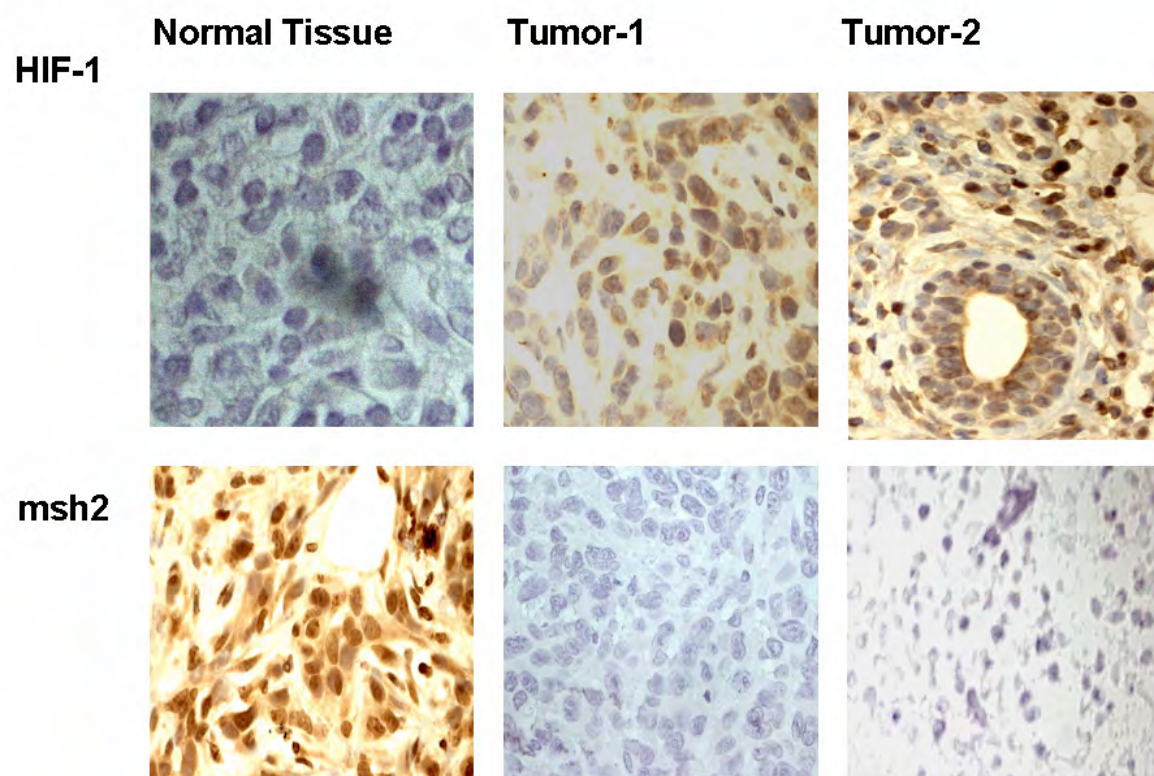


Figure 4: MLH1 and MSH2 mRNA and protein expression

