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14. ABSTRACT

The hypothesis in this project is that homocysteine is an oncometabolite in breast cancer. We propose to test this hypothesis with three specific aims: (1) Investigate using two different mouse models of spontaneous breast cancer (MMTV-HRAS mouse and MMTV-PyMT mouse) whether *Mthfr* is silenced through DNA methylation and as a result the levels of the oncometabolite homocysteine are elevated in tumors; (2) Investigate whether homocysteine promotes breast cancer progression and lung metastasis by comparing the disease process in MMTV-HRAS and MMTV-PyMT mice on two different genetic backgrounds: $Mthfr^{+/+}$ and $Mthfr^{-/-}$. Investigate the ability of homocysteine to induce TGF-β, ANGPTL4, and MMP-9 in breast cancer cell lines and to disrupt the barrier function of lung microvascular endothelial cells; (3) Investigate using breast cancer cell lines whether over expression of MTHFR or exposure to N^5-methyltetrahydrofolate decreases cell proliferation in vitro and suppresses tumor growth in xenografts in vivo.

15. SUBJECT TERMS
Homocysteine, Oncometabolite, Breast cancer, Methylenetetrahydrofolate reductase, Mouse models of breast cancer, Tumor progression , Metastasis to the lung, Breast cancer cell lines, Lung endothelial cells

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Introduction

The goal of the project is to evaluate the validity of the hypothesis that the amino acid homocysteine functions as an oncometabolite in breast cancer. An oncometabolite is defined as a metabolite that is produced specifically in tumor cells and plays a positive role in tumor growth and/or metastasis. Homocysteine is well known for its toxic effects and the plasma levels of this amino acid are widely monitored to assess the risk of cardiovascular disease. This is because homocysteine has deleterious effects on vascular system. Homocysteine is also known for its ability to induce oxidative stress and alter gene expression pattern. It is also an excitotoxin, with the ability to potentiate the signaling pathway associated with the NMDA receptor. The plasma levels of this amino acid are primarily determined by the activities of two enzymes: cystathionine beta-synthase and methionine synthase. Methionine synthase converts homocysteine into methionine using the co-factor N^5-methyltetrahydrofolate. This co-factor is generated by the reduction of N^5,N^{10}-methylenetetrahydro folate by the enzyme methylene tetrahydrofolate reductase (MTHFR). Polymorphisms in MTHFR occur in general population resulting in significant decrease in the catalytic activity of the enzyme; these polymorphisms are associated with an increase in plasma levels of homocysteine. Our hypothesis was that tumor cells accumulate homocysteine by epigenetic silencing of MTHFR and that the resultant increase in homocysteine alters the gene expression pattern in tumor cells in such a manner that promotes tumor growth. In addition, the elevated levels of homocysteine also impact on vascular endothelial cells making them leaky and more permeable. This latter effect promotes extravasation of tumor cells from the primary site and consequently facilitate metastasis, particularly in the lung, one of the primary metastatic sites for breast cancer.

We proposed to examine the validity of the hypothesis with three specific aims:

1. Investigate using mouse models of spontaneous breast cancer whether Mthfr is silenced through DNA methylation and as a result the levels of homocysteine are elevated in tumors.
2. Investigate whether homocysteine promotes breast cancer progression and lung metastasis by comparing the disease progression in a mouse model of spontaneous breast cancer on two different genetic background: $Mthfr^{+/+}$ and $mthfr^{-/-}$. In addition, investigate the ability of homocysteine to induce TGF-beta, ANGPTL-4 and MMP-9 in breast cancer cell lines and also to disrupt the barrier function of lung microvascular endothelial cells.
3. Investigate using breast cancer cell lines whether over-expression of MTHFR or exposure to N^5-methyltetrahydrofolate decreases cell proliferation in vitro and suppresses tumor growth in xenografts in vivo.

Keywords

Homocysteine, Oncometabolite, Breast cancer, Methylenetetrahydrofolate reductase, Mouse models of breast cancer, Tumor progression , Metastasis to the lung, Breast cancer cell lines, Lung endothelial cells, Angiopoietin-like-4, DNA methyltransferase

Accomplishments

Year 1

Expression of Mthfr in mouse breast tumors (completed in Year 1 and included in Year-1 progress report).

We examined the expression of Mthfr in mammary tumors collected from four different mouse models of spontaneous breast cancer: MMTV-Hras-Tg, MMTV-Neu-Tg, MMTV-PyMT-Tg, and C3(1)-SV40-Tg. The expression of mRNA levels was compared with age-matched control mice. Mammary gland from control mice expressed Mthfr mRNA, but the expression was undetectable in breast tumors collected from all four mouse models of spontaneous breast cancer. With the same RNA samples, we assessed the expression of the DNA methyltransferases Dnmt1, Dnmt3a and Dnmt3b to determine if changes in the expression of any of these enzymes correlate with the observed decrease in Mthfr mRNA. We found increased expression of Dnmt1 and Dnmt3b in breast tumors compared to normal mammary gland. The expression of Dnmt3a was not altered.

These data show that the expression of Mthfr is markedly silenced in breast cancer and that this silencing could be due to increased DNA methylation of Mthfr gene because of the increased expression of Dnmt1 and Dnmt3b.

Compare the levels of homocysteine between normal mammary gland and mammary tumors (completed in Year 1 and included in Year-1 progress report).
We measured the tissue levels of homocysteine in mammary tumors collected from two different mouse models of spontaneous breast cancer: MMTV-Hras-Tg and MMTV-PyMT-Tg. We found marked elevation of this amino acid in tumor tissues compared to control tissue. The increase in MMTV-Hras-Tg mouse mammary tumor tissue was 4.5 ± 1.3 fold. The corresponding value in MMTV-PyMT-Tg mouse mammary tumor tissue was 7.3 ± 1.8 fold. These data show that the decrease in the expression of Mthfr in mammary tumor tissues in these two mouse models of spontaneous breast cancer correlates with a parallel increase in tissue levels of homocysteine. This is expected because Mthfr is one of the two enzymes that control the levels of homocysteine.

Year 2

Monitor tumor incidence, metastasis and survival time in MMTV-PyMT/Mthfr$^{+/+}$ and MMTV-PyMT/Mthfr$^{-/-}$ mice (completed in Year 2 and included in Year-2 progress report).
We generated MMTV-PyMT/Mthfr$^{+/+}$ and MMTV-PyMT/Mthfr$^{-/-}$ mice by crossbreeding MMTV-PyMT-Tg mice with Mthfr$^{-/-}$ mice and confirmed the genotype by PCR. We then monitored the incidence and progression of mammary tumors in both mouse lines. The average age at which tumors appeared in MMTV-PyMT/Mthfr$^{+/+}$ mice was 91 ± 4 days. In contrast, tumors appeared at an earlier age in MMTV-PyMT/Mthfr$^{-/-}$ mice (68 ± 4 days) (Fig. 1). The difference between the two values was statistically significant ($P < 0.01$). The tumors were allowed to grow in both groups and when the mice became moribund, they were killed and the tumor tissues were collected and processed for immunostaining, preparation of protein lysates for western blotting, and preparation of RNA for qPCR. Lungs were perfused with India ink and Fekete solution to visualize metastatic nodules. We found more metastatic nodules in MMTV-PyMT/Mthfr$^{-/-}$ mice than in MMTV-PyMT/Mthfr$^{+/+}$ mice (Fig. 1). On an average, there were 3 nodules in MMTV-PyMT/Mthfr$^{+/+}$ mouse lungs in contrast to 7 nodules in MMTV-PyMT/Mthfr$^{-/-}$ mice (Fig. 1).

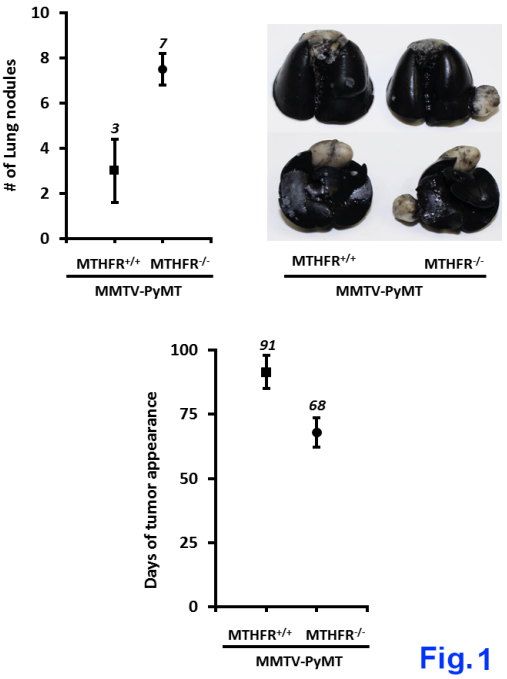


Fig. 1

Monitor the levels of homocysteine in mammary tumors in PyMT/Mthfr$^{+/+}$ and MMTV-PyMT/Mthfr$^{-/-}$ mice (completed in Year 2 and included in Year-2 progress report).
We collected the tumors from MMTV-PyMT/Mthfr$^{+/+}$ and MMTV-PyMT/Mthfr$^{-/-}$ mice and measured the tissue levels of the amino acid homocysteine. The tissue concentration of homocysteine in breast tumors obtained from MMTV-PyMT mice on intact Mthfr genetic background was 13.4 ± 2.3 μM. This value was not significantly different from the tissue levels of this amino acid in non-cancerous mammary glands obtained from MMTV-PyMT mice at 2 months of age when there were no visible tumors (11.7 ± 3.2 μM). However, the levels of homocysteine were significantly higher in tumors obtained from MMTV-PyMT transgenic mice on Mthfr-null background (93.6 ± 11.9 μM). Thus, there was a ~7-fold increase in the tissue levels of homocysteine in breast tumors in MMTV-PyMT transgenic mice when on Mthfr-null background than on Mthfr$^{+/+}$ background.

The occurrence of similar levels of homocysteine in breast tumors versus normal mammary gland with intact Mthfr came as a surprise because we expected downregulation of Mthfr in mammary tumors so as to generate the oncometabolite homocysteine. This however was not the case. A possible explanation is that even though there were no visible tumors in 2-month-old MMTV-PyMT-Tg mouse mammary glands, these tissues are not "normal" because they do express the oncogene PyMT. Therefore, it is likely that the levels of homocysteine are already elevated in mammary tissues from this mouse line even when there are no tumors. The tissue levels of homocysteine in mammary tissues from normal mouse are very low, in the range of 4-5 μM. The levels of this amino acid in mammary tumors collected from MMTV-PyMT mice are at least 2-to-3-fold higher than this normal levels. The data show that homocysteine levels do increase in mammary tumors from the MMTV-PyMT mouse model of spontaneous breast cancer.

Analyze the role of homocysteine on expression of TGF-β, ANGPTL-4 and MMP-9 in breast cancer cell lines (completed in Year 2 and included in Year-2 progress report).
We cultured the human breast cancer cell lines MCF7 and MB231 in the presence (500 μM) and absence of homocysteine for three passages (chronic exposure to homocysteine in the form of homocysteine thiolactone, HTL) and then prepared total RNA from the cells to assess the expression of TGF-β, ANGPTL-4, and MMP-9 by RT-PCR. We found that treatment with homocysteine enhanced the expression of all three genes that are involved in tumor promotion (TGF-β) and metastasis (ANGPTL-4 and MMP-9) in both cell lines.

Analyze the influence of homocysteine and ANGPTL-4 on the permeability of lung microvascular endothelial cells (completed in Year 2 and included in Year-2 progress report).

Lung is the one of the primary sites for breast cancer metastasis, and it is believed that the permeability of lung microvascular endothelial cells plays a critical role in metastasis by allowing the circulating cancer cells to seed the lung parenchymal tissue. ANGPTL-4 has been shown to increase the permeability of lung endothelial cells and this protein is expressed at higher levels in breast cancer cells. Our data show that exposure of breast cancer cells to homocysteine increases the expression of ANGPTL-4. Therefore, we hypothesized that homocysteine promotes lung metastasis of breast cancer by increasing the expression of ANGPTL-4 in tumor cells and possibly also in lung endothelial cells, consequently increasing the permeability of these cells. To test this hypothesis, we examined the effects of homocysteine and recombinant ANGPTL-4 on the permeability of lung microvascular endothelial cells in vitro. For this, we used two different experimental approaches. First, we used the Electric Cell-substrate Impedance Sensing (ECIS) system to measure the impedance of the monolayers of lung endothelial cells with and without exposure to homocysteine or recombinant ANGPTL-4. The impedance is inversely proportional to permeability of the monolayer. With this system, we found homocysteine as well as ANGPTL-4 decreased the impedance of these cells, thus indicating increased permeability of the monolayer. In the second approach, we cultured the endothelial cells on a porous filter in a Transwell culture system in the presence or absence of homocysteine or ANGPTL-4 and periodically monitored the transcellular electrical resistance (TER). We found this resistance to decrease by 35 ± 6 % (N = 6; $p < 0.01$) when the cells were cultured in the presence of homocysteine (250 μM) and ANGPTL-4 (50 μg/ml). The decrease in TER is an indication of increased permeability. We corroborated this data with the transcellular transfer of the macromolecule FITC-dextran, which increased by 24 ± 4 % and 38 ± 7 %, respectively in cells exposed to homocysteine (250 μM) and ANGPTL-4 (50 μg/ml). In both cases, the difference was statistically significant ($p < 0.01$).

Year 3

Analyze the breast tumor tissues from PyMT-mice with and without Mthfr deletion (completed in Year 3)

We found significant differences in the growth and lung metastasis of breast tumors in MMTV-PyMT-transgenic mice depending on whether or not Mthfr was deleted. This implied that the presence or absence of elevated levels of homocysteine impacted on the growth and metastatic profile of breast tumors in mice. We then analyzed the tumor tissues from Mthfr-positive and Mthfr-negative mice to examine the differences at the molecular and cell biological levels. First, we compared the expression of various genes related to angiogenesis and lung metastasis. We found the expression of metalloproteinase-9 (MMP9), angiopoietin-like 4 (ANGPTL-4), vascular endothelial growth factor A (VEGFA) and E-cadherin is increased in Mthfr-negative tumors compared to Mthfr-positive tumors (Fig. 2). The increase in ANGPTL-4 and VEGFA is statistically significant. ANGPTL-4 is known to increase the permeability of lung microvascular endothelial cells; this has been demonstrated by our group (Year 2 progress report) as well as by other investigators. The increased incidence of lung metastasis of breat tumors in PyMT-mice in association with Mthfr deletion could therefore be explained on the basis of increased expression of ANGPTL-4. VEGFA is an important growth factor for vascular endothelial cells which promotes angiogenesis and

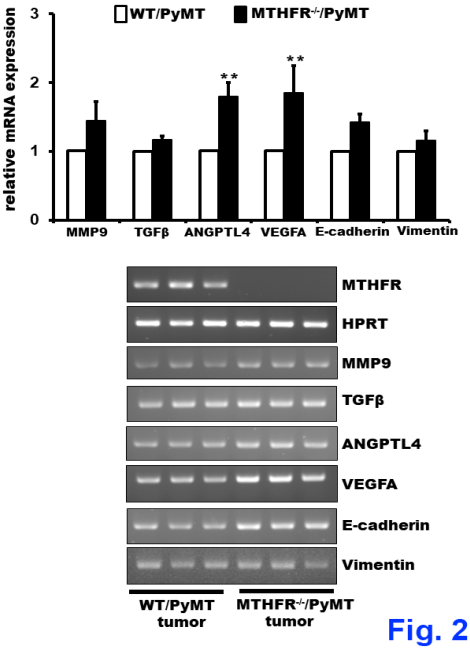


Fig. 2

supports tumor growth. The increased incidence and growth of breast tumors in PyMT-mice in association with Mthfr deletion would be at least partly explained on the basis of increased expression of VEGFA.

We also examined the proliferation index of breast tumors in PyMT-mice with and without Mthfr deletion in tissue sections using immunohistochemistry for the proliferation marker Ki67 (Fig. 3). We found robust increase in Ki67 in Mthfr-negative tumors compared to Mthfr-positive tumors. The same was true with metastasized lung nodules. This suggests that the presence of elevated levels of homocysteine provides a proliferative advantage to breast tumors in PyMT-mice.

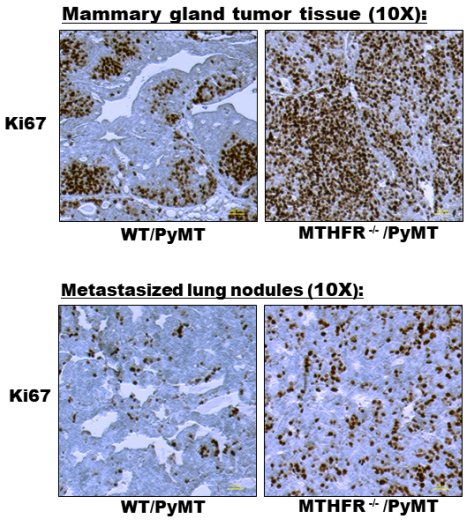
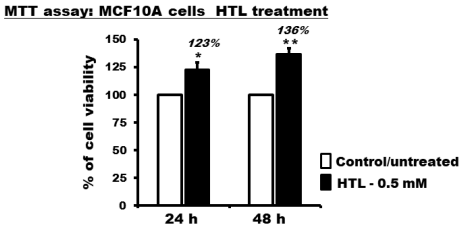


Fig. 3

Impact of chronic exposure to homocysteine on the morphology and proliferation of normal mammary epithelial cells in vitro (completed in year 3)

To corroborate the in vivo data on the impact of elevated levels of homocysteine on the growth and metastasis of breast tumors in mice, we performed in vitro experiments to examine the impact of chronic exposure to homocysteine on normal mammary epithelial cells. We cultured MCF10A cells, which represent normal human breast epithelium, in the presence of 0.5 mM homocysteine thiolactone for five passages to mimic chronic exposure to excess homocysteine. We then examined the proliferation of the cells using the MTT assay and also examined the cell morphology using the phase-contrast microscopy. We found a significantly increased proliferation rate of the cells in response to chronic exposure to homocysteine (Fig. 4). Even more importantly, we noticed a significant change in morphology of the cells. Homocysteine-exposed cells were transformed into elongated, spindle-shaped cells, a clear indication of epithelial-to-mesenchymal transition (Fig. 4). The increased proliferation rate correlates with the increased tumor growth in vivo and the epithelial-to-mesenchymal transition correlates with increased tendency for metastasis.



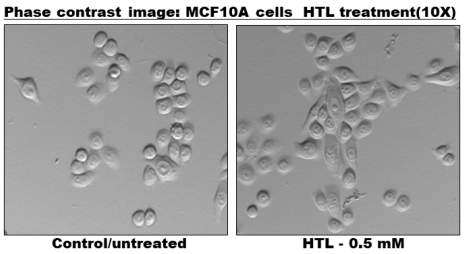


Fig. 4

Synopsis of accomplishments for the entire project period

- Mthfr is silenced to a significant extent in mouse models of spontaneous breast cancer.
- Homocysteine levels are indeed increased in breast tumor tissues from mouse models of spontaneous breast cancer.
- Successful generation of MMTV-PyMT/Mthfr$^{+/+}$, MMTV-PyMT/Mthfr$^{-/-}$, MMTV-PyMT/Mthfr$^{+/+}$ and MMTV-PyMT/Mthfr$^{-/-}$ mouse lines.
- Deletion of Mthfr promotes breast cancer growth and lung metastasis in MMTV-PyMT mice.
- Demonstration of the association between Mthfr deletion and elevated levels of homocysteine in breast tumors in mice.
- Homocysteine and ANGPTL-4 render lung microvascular endothelial cells more permeable, thus potentially facilitating the transfer of circulating tumor cells across the blood vessel into lung parenchymal tissue for effective seeding, thus facilitating lung metastasis.
- There is increased expression of the angiogenic factor VEGFA and the endothelial cell layer permeabilizing factor ANGPTL4 in PyMT-driven breast tumors with concomitant absence of Mthfr.
- The proliferation rate of Mthfr-null breast tumors in PyMT-mice is much greater than that of Mthfr-positive breast tumors in PyMT-mice.
- Chronic exposure of normal human mammary epithelial cells to homocysteine promotes epithelial-to-mesenchymal transition and also facilitates cell proliferation.

Impact

Increased circulating levels of homocysteine are widely considered as a risk factor for cardiovascular disease because of the deleterious effects of this amino acid on vascular endothelial cells. MTHFR (methylene tetrahydrofolate reductase) is an enzyme which plays a critical role as a determinant of homocysteine levels. For the conversion of homocysteine into methionine, N^5-methyltetrahydrofolate serves as a cofactor along with vitamin B12. N^5-methyltetrahydrofolate is generated from N^5,N^{10}-methylenetetrahydrofolate by MTHFR. There are polymorphisms in this enzyme that result in impairment of catalytic activity; such polymorphisms are associated with increased levels of homocysteine. We initiated the current project with the hypothesis that homocysteine is also an oncometabolite for breast cancer that promotes tumor growth at the primary site and also promotes metastasis of the cancer in the lung. The idea was that MTHFR is silenced in breast cancer due to epigenetic changes, which elevates the levels of homocysteine. Our studies have shown that Mthfr is indeed silenced to a significant extent in breast cancer. We then used a mouse model of spontaneous breast cancer, namely MMTV-PyMT transgenic mouse. These mice were crossbred with $Mthfr^{-/-}$ mice. Deletion of Mthfr in MMTV-PyMT mice increases the tissue levels of homocysteine in mammary gland. Even though this elevated homocysteine levels are not sufficient in itself to initiate tumorigenesis, the increase in the levels of this amino acid due to deletion of the enzyme Mthfr promotes tumor growth at the primary site, accelerates the process of carcinogenesis, and facilitates lung metastasis in a spontaneous mouse model of breast cancer. Analysis of gene expression pattern in tumors from MMTV-PyMT mice with $Mthfr^{+/+}$ and $Mthfr^{-/-}$ genetic background has revealed significant alterations in the transcriptome profiles, which corroborates the increased tumor growth and lung metastasis as a result of Mthfr deletion. These findings suggest that monitoring the circulating levels of homocysteine in breast cancer patients might be of prognostic value to identify the patients who are likely to exhibit an aggressive tumor growth and increased risk for lung metastasis.

Changes/Problems

We were able to complete most of the tasks approved in the Statement of Work for the entire three-year project. The only task that we were unable to complete is the evaluation of the effect of N^5-methyltetrahydrofolate administration on tumor growth in xenografts in vivo. This was due to the re-location of the principal investigator's laboratory from the Medical College of Georgia, Augusta, GA to the Texas Tech University Health sciences Center, Lubbock, TX. The grant was funded when the principal investigator was at the Medical college of Georgia and the first year of the project was completed there at the original award site. Then in September 2014, the principal investigator moved to his current location. Because of this change in the performance site, the award was suspended temporarily and then re-initiated on 01/01/2016. The transfer of the $Mthfr^{-/-}$ mice from the Medical College of Georgia to the Texas Tech University Health Sciences Center took more than 8 months because of the rigid policies at the current institution regarding the health report of the mice. Therefore, we had to re-derive the mice after sending the founders from the Medical College of Georgia to Charles River and the re-derived mice were then transferred to the Texas Tech University Health Sciences Center. Because of this long, but unexpected, delay in obtaining the mice, we were not able to complete the task related to the in vivo studies to examine the efficacy of treatment with N^5-methyltetrahydrofolate.

Products

The data on the promotion of breast cancer growth and lung metastasis by Mthfr deletion are being finalized for preparation of a manuscript for publication.

Participants and other Collaborating Institutions

Texas Tech University Health Sciences Center

Special Reporting Requirements

None

Appendices

None