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Award Number: W81XWH-10-1-0416

TITLE: Nonapoptotic Function of Caspase-6 in Promoting Mammary Carcinogenesis

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REPORT DATE: January 2012

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for public release; distribution unlimited

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REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

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1. REPORT DATE (DD-MM-YYYY) 01-01-2012		2. REPORT TYPE Final		3. DATES COVERED (From - To) 15 SEP 2010 - 14 DEC 2011	
4. TITLE AND SUBTITLE Nonapoptotic Function of Caspase-6 in Promoting Mammary Carcinogenesis				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER W81XWH-10-1-0416	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Dr. Xiangxi Xu E-Mail: xxu2@med.miami.edu				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) University of Miami Miami, FL 33136				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT Abstract on next page.					
15. SUBJECT TERMS nuclear envelope, nesprin/SYNE, nuclear morphology, nuclear deformation, cellular malleability metastasis.					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			19b. TELEPHONE NUMBER (include area code)
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14. ABSTRACT

Malignancy is defined as the elevated mobility and invasiveness of tumor cells, and a deformed nuclear morphology is a common feature of malignant cells. We hypothesized that the decrease or absence of nesprin-1 in breast cancer cells may account for the malignant features of the neoplastic cells, the deformed nuclear morphology and invasiveness/high motility. We propose a pilot study to test this hypothesis.

Indeed, we found that nesprin-1 expression is commonly lost in malignant breast cancer cell lines (**Aim 1**). We found that the suppression of nesprin-1 by siRNA led to nuclear morphological deformation and increased invasion (**Aim 2**). We also tested restoration of nesprin-1 expression in malignant breast cancer cells and nesprin-1 was not sufficiently stable to produce other significant phenotypes in the transfected cells, likely due to technical limitation (**Aim 3**).

The results of these pilot experiments support the initial hypothesis of nesprin-1 as a metastatic suppressor gene and as an underlying link between two prominent features of a malignant cell, nuclear deformation and cellular malleability. We also realize further complexity of nesprin-1 function in breast cancer suppression. The pilot study promotes us to seek further investigation into the role of nesprin-1 in cancer malignancy.

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INTRODUCTION:

Caspases are a family of proteolytic enzymes functions in regulating the cellular activity leading to apoptosis, the phenomenon of cell suicide, or known as program cell death (1). Cancer cells are known to be refractory in the apoptotic program, which allows excessive cell survival and cancer development. Surprisingly, it has been reported that while expression of caspase-3 is reduced, expression of caspase-6 is increased in more than 60% of breast cancer cases (2). Caspase-6 is a p53-inducible gene, but activation of caspase-6 is not sufficient to initiate the apoptotic program; instead it lowers the threshold for additional apoptotic stimulation (1). Overexpression of caspase-6 in cells leads to self-catalyzed activation; however, the activation of caspase-6 does not lead to cell apoptosis, but reduces nuclear lamin A/C, the main substrate for caspase-6 (3). A reduction of lamin A/C is common in cancers, including breast cancer (4). Reduction of lamin A leads to enhanced MAPK activation (5), altered chromatin organization and gene expression (4), increased genomic instability (4), and deformed nuclear morphology (4), which are characteristics of breast cancer cells.

We propose that pathological factors such as inflammation or excessive hormonal stimulation induce caspase-6 activation without activating the apoptotic program in mammary epithelial cells. An increased caspase-6 activity will then degrade and reduce lamin A, leading to growth signal activation, genomic instability, and deformed nuclear morphology that are characteristics of breast cancer cells. The increased growth and genetic changes will lead to the clonal selection and transformation of mammary epithelial cells to develop cancer. We have postulated that the increased growth and genetic changes due to the increased caspase-6 will lead to changes required for breast cells to become cancer.

To test this idea, we propose the following specific aims in a pilot project:

Aim 1. We will determine/confirm if caspase-6 is commonly overexpressed or activated in breast cancer cell lines and primary human breast cancer cells, correlating with level of lamin A protein and aneuploidy.

Aim 2. We will determine if suppression (by siRNA and chemical inhibitors) of caspase-6 will restore lamin A protein level, reduce MAPK activation, and suppress growth in breast cancer cells.

Aim 3. We will test if an increased caspase-6 activity (by transfection of cDNA) is sufficient to induce an enhanced MAPK activation, cell proliferation, and genomic instability in mammary epithelial cells.

In the work completed last year, we have confirmed that caspase-6 expression is commonly increased in breast cancer tissues by immunostaining. We have also found that Caspase-6 activity is commonly increased in breast cancer cells using an activity assay. In some but not all cells, we found that expression of caspase-6 correlates with the reduction of lamin A/C proteins. These pilot experiments have supported the potential role of caspase-6 in the promotion and development of breast cancer.

BODY:

In the last year, we have followed the research plan described in the Statement of Work (SOW). We have accomplished most of the experiments proposed and reached the conclusion to support our hypothesis that increased caspase-6 activity contributes to progression of breast cancer. We produced interesting results that encourage us to seek further study in the future. The works, results, and conclusions are detailed below for each item and task in the SOW.

A series of tasks will be undertaken to accomplish the 3 aims in this proposal.

Aim 1. We will determine/confirm if caspase-6 is commonly overexpressed or activated in breast cancer cell lines and primary human breast cancer cells, correlating with reduction of lamin A protein and aneuploidy.

Aim 2. We will determine if suppression (by siRNA and chemical inhibitors) of caspase-6 will restore lamin A protein level and suppress growth in breast cancer cells.

Aim 3. We will test if an increased caspase-6 activity (by transfection of cDNA) is sufficient to induce cell proliferation and genomic instability in mammary epithelial cells.

Task 1 (month 1-2): Collect 15 breast cancer cell lines and 3 non-cancer human mammary epithelial cell lines. Grow and expand these cells in cell culture, isolate cell lysate and mRNA for further analysis.

Task 2 (month 2): Use the mRNA and cell lysate prepared to perform qRT-PCR and Western blot to measure expression of caspase-6 and lamin A. The activity of caspase-6 will be assayed in cell lysates. We will find out if caspase-6 is commonly overexpressed or activated in breast cancer cells compared to non-cancer mammary epithelial cells, and if the activated caspase-6 reduces its substrate lamin A.

Task 3 (month 3): Collect around 100 breast cancer samples in tissue microarray. Perform immunostaining for lamin A and caspase-6. Antibodies to both proteins are commercially available and of high quality. We will be able to determine if caspase-6 is overexpressed or activated in breast cancer tissues, and if the activity is inversely correlated with the presence of substrate lamin A.

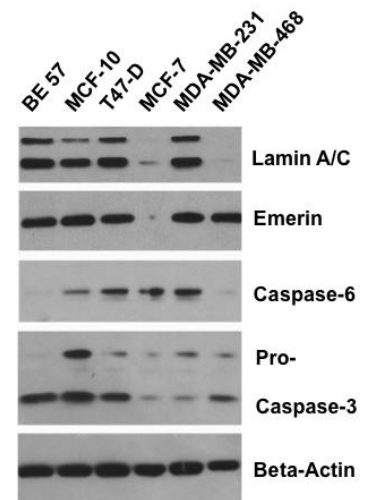
Milestone 1: Task 1-3 (Aim 1) will enable us to verify if caspase-6 is commonly over-expressed or activated in breast cancer cells, and if activation of caspase-6 is able to reduce the cellular level of its substrate lamin A.

We started the project by testing commercially available antibodies for Western blot analysis of caspase-6, comparing to apoptotic caspase-3, and potential substrates lamin A/C and emerin. We used a primary cell preparation of human mammary epithelial cells as a baseline control. In several breast cancer cell lines (T47-D, MCF-7, MDA-MB-231, MDA-MB-468) tested, we observed higher expression of caspase-6 in cancer lines T47-D, MCF-7, MDA-MB-231, but not in MDA-MB-468 (**Figure 1**). The primary breast epithelial cells (BE 57) shows little caspase-6. Activated caspase-3 is high in non-cancer cells BE 57 and MCF-10, and lower in cancer cells MCF-7, MDA-MB-231, and MDA-MB-468. Thus, as expected, caspase-6 is generally increased and caspase-3 (activity) is reduced in cancer cells. However, the caspase-6 expression is not generally correlated with the level of substrates lamin A/C and emerin.

Figure 1. Expression of caspase-6 in breast epithelial cells and cancer cell lines: Western blot.

Primary human mammary epithelial cells (BE 57), non-tumorigenic immortalized breast epithelial cells (MCF-7), and breast cancer cell lines (T47-D, MCF-7, MDA-MB-231, MDA-MB-468) were grown and cell lysate was analyzed by Western blot for caspase-6, comparing to additional markers including caspase-3 (both pro-enzyme and cleaved active protease), lamin A/C, emerin, and beta-actin (protein loading control).

An anti-caspase-6 antibody (rabbit IgG from Sigma) recognizes a 34 kD band corresponding to the caspase-6 precursor.



We further investigated the activity of caspase-6 in the cells, since the Western blot of caspase-6 showed protein level, but did not show the amount of activated form. The activity was measured by a biochemical assay using the chromogenic substrate Ac-VEID-AFC. In the first experiment, we documented that in three available primary cell preparations of human mammary epithelial cells, the caspase-6 activity is lower than breast cancer cells MDA-MB-468 (**Figure 2**). In subsequent analysis, we assayed a panel of both breast and ovarian epithelial and cancer cells (**Figure 3**). We concluded that although there were some variations (both experimental and biological), most cancer cells generally have an increased caspase-6 expression and activity.

Figure 2. Activity assay of caspase-6 in breast epithelial cells and cancer cell lines. Four available primary human mammary epithelial cells (BE 0, BEC 300, BEC 500, BEC 1000) were compared with breast cancer cells MDA-MB-468 for caspase-6 activity. The activity was measured by a biochemical assay using the chromogenic substrate Ac-VEID-AFC, and the product was measured for optical absorption at 400 nm. The value of absorbance correlates with caspase-6 activity.

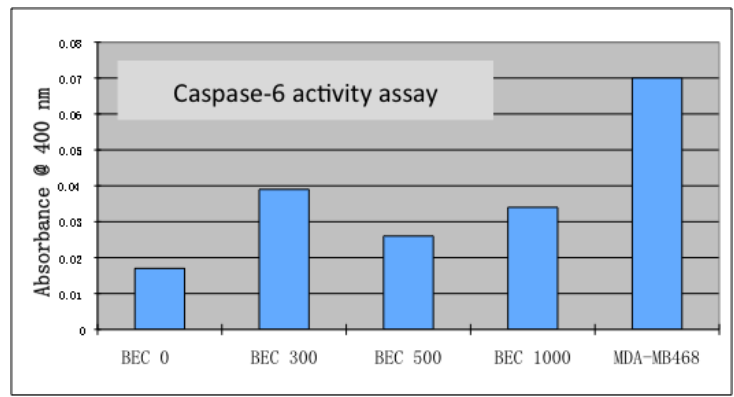
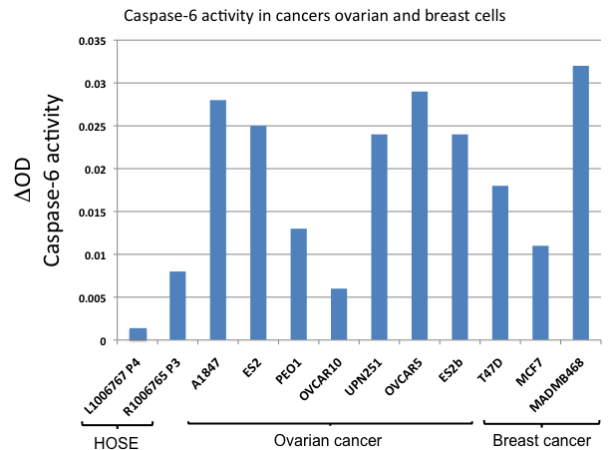


Figure 3. Activity assay of caspase-6 in breast and ovarian epithelial cells and cancer cell lines. Available primary human ovarian surface epithelial cells (HOSE), ovarian cancer cells (A1847, ES2, PEO1, OVACAR10, UPN251, OVCAR5, ES2-b), breast cancer cells (T47-D, MCF-7, MDA-MB-468), were analyzed for caspase-6 activity.

Cell lysate was incubated with VEID-AFC substrates in according to the manufacturer's protocol (G-Biosciences). Following incubation at 37°C for 30 min to 2 hours to reach optimal color reaction, the absorbance at 400 nm was measured.



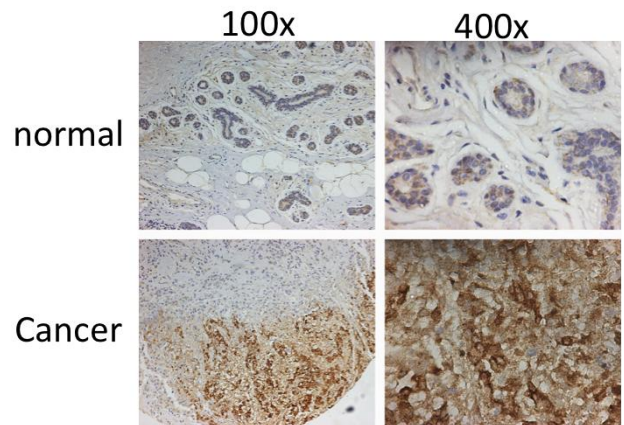
Additionally, we proceeded to measured caspase-6 and caspase-3 expression in human breast tissues and tumors by immunohistochemistry in a tissue array containing about 300 tissues (some were not informative due to cutting problem in each individual slide). Following some testing to optimizing the procedure, we concluded that we have been able to determine the expression of caspase-6 and caspase-3 in archived human tissues. As shown by representative examples (**Figure 4**), normal human mammary tissues show little caspase-6 staining and about 60% of cancer tissues showed strong caspase-6 staining (**Figure 4**).

Figure 4. Examples of immunostaining of caspase-6 in normal breast and cancer tissues.

Available cancer tissue arrays (from Fox Chase Cancer Center) were stained for caspase-6. The included one of the three normal mammary tissues (upper panels) contains little caspase-6 staining. The cancer (lower panel) is highly positive of caspase-6 staining. A 100X magnification (left panels) and a 400X magnification are shown.

About 60% of cancer tissues show strong positive staining for caspase-6.

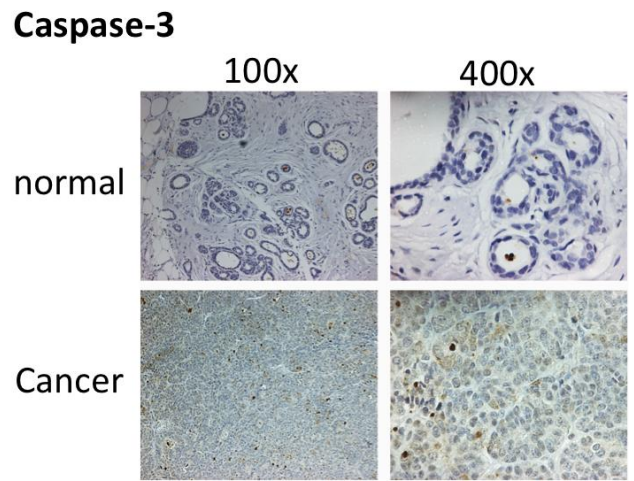
Caspase-6



For caspase-3, the staining is mostly weak or negative in all cancer or normal mammary tissues, as shown by examples in **Figure 5**. A small percentage of strongly stained nuclei were observed in both normal and cancer tissues. The caspase-3 positive cells are likely apoptotic cells.

Figure 5. Examples of immunostaining of caspase-3 in normal breast and cancer tissues.

Available cancer tissue arrays (from Fox Chase Cancer Center) were stained for caspase-3. Both the included normal mammary tissues (upper panels) and the cancer (lower panel) contain little caspase-3 staining, except for occasional positive nucleus of apoptotic cells. A 100X magnification (left panels) and a 400X magnification are shown.



These tasks have enabled us to conclude that caspase-6 expression and activity are increased in cancer over normal in both cells and tissues. In contrast, cancer cells and tissues have a reduced caspase-3 expression and activity. However, generally, the expression of caspase-6 and potential substrates lamin A/C and emerin, as assayed, is not inversely correlated.

Task 4 (month 4-6): siRNA will be prepared to target caspase-6, and at the least two effective sequences will be used. A panel of breast cancer cells identified in Aim 1 that have high caspase-6 activity and low lamin A level, will be transfected with siRNA to target caspase-6. The transfected cells will be analyzed for caspase-6 activity by biochemical assay, lamin A level by Western blot, MAPK activity by Western blot with phospho-specific antibodies, and cell growth by counting and MTT assay.

Task 5 (month 6): The siRNA transfected cells will be analyzed for nuclear morphology by immunofluorescence microscopy, to observe if the deformed cancer cell nuclear morphology will be reverted to round and smooth shape.

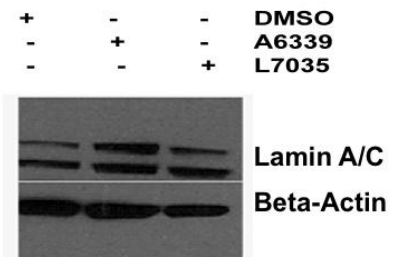
Milestone 2: These experiments in Task 4 and 5 (Aim 2) will determine/confirm if down regulation of caspase-6 is sufficient to restore lamin A level, and also reverts the deformed nuclear morphology of cancer cells to smooth oval shape.

In several experiments testing the efficacy of siRNA to down regulate caspase-6, we were not successful to identify an efficient siRNA sequence. We speculated that the technical problem was due to our ability to accurately assay caspase-6 protein level, as well as the possibility of slow turn over rate of caspase-6. We decided that a better approach may be the use of caspase-6 null cells from the knockout mice. This is a viable approach that we are seeking to develop in the future.

In an alternative approach, we decided to use available caspase-6 chemical inhibitors to test the consequence of suppressing the activity. In a preliminary experiment (**Figure 6**), the inhibition of either caspase-6 or proteasome was found to increase lamin A/C protein, with higher increase in caspase-6 inhibition.

Figure 6. Effect of protease inhibition on lamin A/C protein level.

Breast cancer cells were treated with DMSO (solvent control), caspase-6 inhibitor A6339 (N-Acetyl-Val Glu_Ile-Asp-Al, from Sigma), or Calpain and Proteasome Inhibitors L7035 (clasto-lactacystin β -lactone, cell-permeable and irreversible inhibitor, from Sigma). The cell lysate was harvested to assay lamin A/C protein level by Western blot.

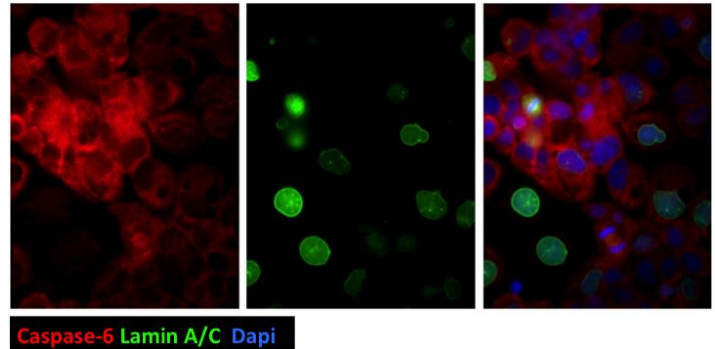


Another mean to analyze the protease-substrate relationship between caspase-6 and lamin A/C is to examine their correlation in individual cells. We used immunofluorescence microscopy to assay caspase-6 and lamin A/C simultaneously (**Figure 7**). We observed that the expression both the caspase-6 and lamin A/C is heterogeneous within a cancer cell population. A correlation was observed that cells with high nuclear lamin A/C expression are often lower in caspase-6 expression (**Figure 7**). Additionally, cell with high lamin A/C expression are generally round and smooth. Although, subsequent analysis suggest that the correlation is true only in some but is not general true in all cell lines.

Figure 7. Immunofluorescence microscopy of caspase-6 and lamin A/C in cancer cells.

Cancer cells were stained for caspase-6 (red), lamin A/C (green), and DAPI (blue) in indirect immunofluorescence microscopy..

Note that the expression of lamin A/C is inversely correlates with the expression of caspase-6 in individual cells.



Task 6 (month 4-8): In 3 lines of primary mammary epithelial cells, activated caspase-6 will be transfected and expressed. The transfected clones will be selected by neo-resistance and the clones will be expanded.

Task 7 (month 9-11): The caspase-6 expressing cells will be assayed for caspase 6 activity by biochemical assay, lamin A level by Western blot, MAPK activity by Western blot with phospho-specific antibodies, cell growth by counting and MTT assay, and nuclear morphology by immunofluorescence microscopy, compared to vector-transfected cells.

Task 8 (month 11-12): The selected caspase-6-expressing cells will be expanded and subjected to cytogenetic analysis by counting the chromosome number of 50 metaphase spreads, comparing with vector-transfected cells.

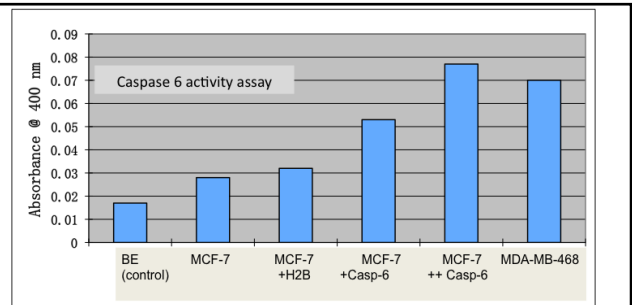
Task 9 (month 11-12): The selected caspase-6 expressing cells will be expanded and subjected to genomic analysis by CGH, comparing with vector-transfected cells.

Milestone 3: Task 6-9 will accomplish Aim 3: To verify if the activation of caspase-6 is sufficient to induce chromosomal and genomic instability. The caspase 6-activated cells will have a diverse chromosome composition in the 50 metaphase spreads because of chromosomal instability of the cells. The CGH also will provide information about genetic changes of the cells.

We started by construction of a caspase-6 expression vector and tested the expression and activity in cells. By activity assay, we observed a dose dependent increased in caspase-6 activity in transfected cells, suggesting the success in transfection and expression of caspase-6 (**Figure 8**).

Figure 8. Activity assay of caspase-6 in transfected cells.

Caspase-6 expression vector was transfected into MCF-7 (+, 1 µg; ++, 4 µg). Histone H2B expression construct was used as a transfection control. Caspase-6 activity was measured using the chromogenic substrate Ac-VEID-AFC.

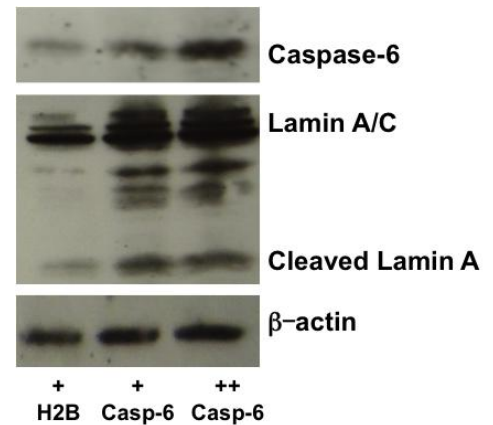


The transfected and expressed caspase-6 was also analyzed using Western blotting (**Figure 9**). In three experiments with similar result, the expression of caspase-6 was observed in a dose dependent manner as assayed by Western blot. Surprisingly, we observed that the expression of caspase-6 induced an increased, instead of an expected decrease, in the amount of lamin A/C (**Figure 9**). Some far, we have no feasible

explanation. However, we believe that the unexpected observation is highly interesting. This may imply a regulatory role of caspase-6 expression in lamin A/C expression. We will seek future support to confirm and investigate the possible link between caspase-6 activity and lamin A/C proteins.

Figure 9. Western blot analysis of caspase-6 in transfected cells.

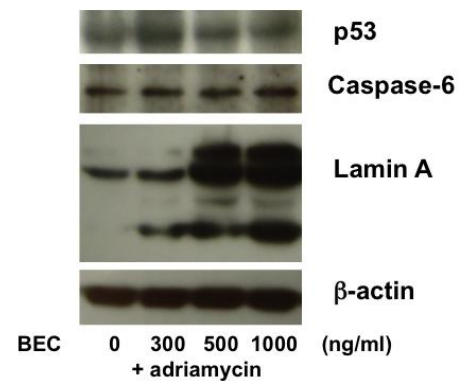
Caspase-6 expression vector was transfected into MCF-7 (+, 1 μ g; ++, 4 μ g). Histone H2B expression construct was used as a transfection control. Caspase-6 activity was measured using Western blot. The corresponding lamin A/C amount was also determined.



We further determined that the transfection efficiency and the expression of caspase-6 is only around 5% in primary mammary epithelial cells. The low transfection efficiency prevented us to properly analyze the phenotypes of caspase-6 expression. However, we used an alternative approach, by induce p53 and then caspase-6 by adriamycin. In this experiment, primary human mammary epithelial cells were treated for adriamycin 0, 300, 500, and 1000 ng/ml for 6 hours. The cells were then harvested for Western blot analysis of p53, caspase-6, lamin A, and beta-actin (**Figure 10**). We observed that adriamycin induced an increase in p53 level, and was highest at the 300 ng/ml dosage. Caspase-6 level was not significantly induced in the primary mammary epithelial cells. However, lamin A protein level was greatly increased when the cells were treated with 500 and 1000 ng/ml of adriamycin.

Figure 10. Western blot analysis of adriamycin-treated primary mammary epithelial cells.

Primary human mammary epithelial cells were treated for adriamycin 0, 300, 500, and 1000 ng/ml for 6 hours. The cells were then harvested for Western blot analysis of p53, caspase-6, lamin A, and beta-actin.



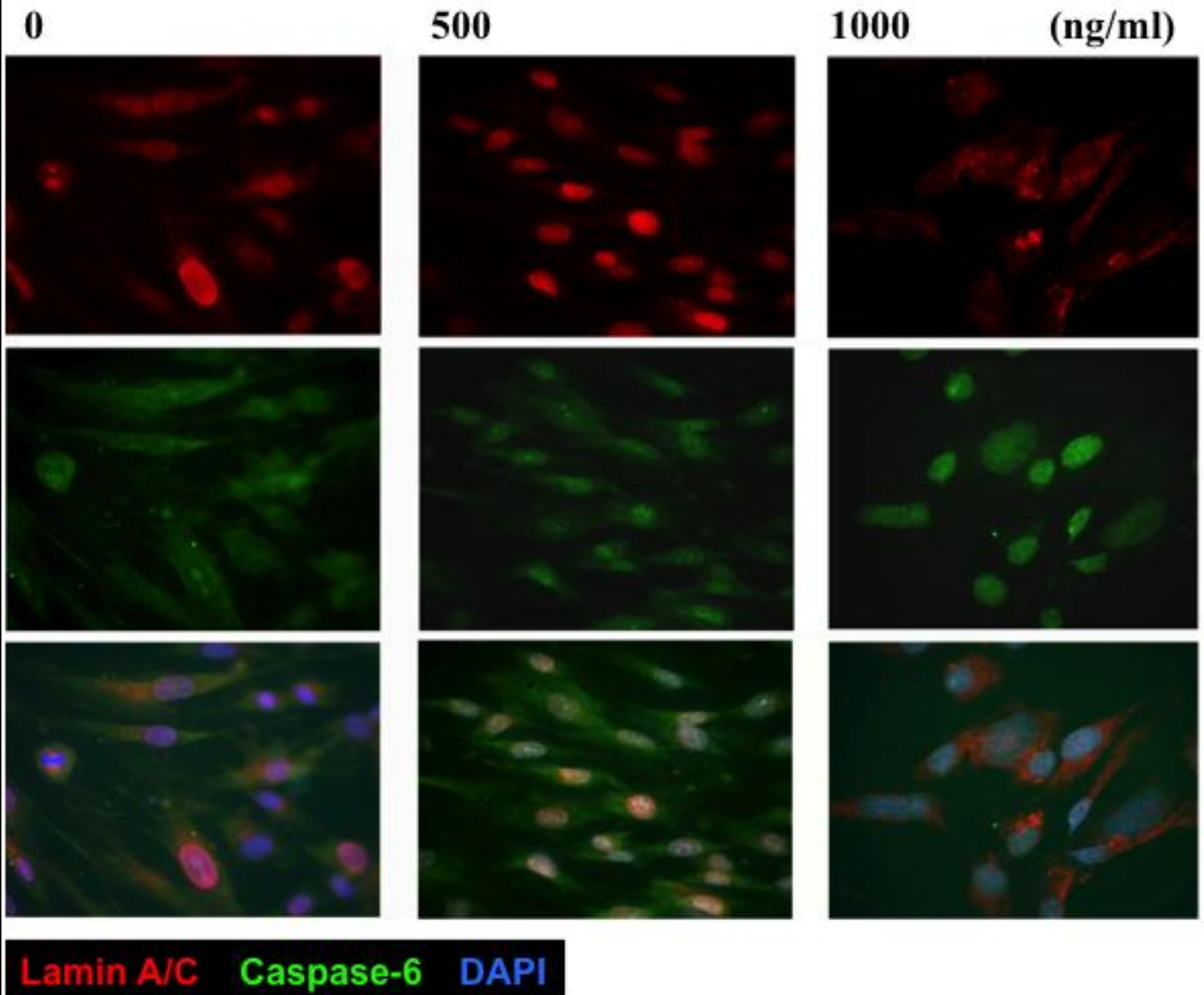
Again, we do not yet have a good explanation of the induction of lamin A/C protein level by adriamycin. It is possible that adriamycin induces p53, which in term induced caspase-6 activation (not expression level). This is consistent with the earlier observation that caspase-6 induces an increase in lamin A/C protein level.

The induced increase of lamin A/C expression is interesting, and we have followed up with additional experiments. In one experiment, we treated the cells with adriamycin and analyzed the expression of lamin A/C and caspase-6 using immunofluorescence microscopy (**Figure 11**). We observed that normally, caspase-6 was cytoplasmic localized. However, increasingly high adriamycin induced transition to a nuclear localization of caspase-6. At high adriamycin level, lamin A/C was not longer nuclear but became cytoplasmic (**Figure 11**). These observations are highly interesting and warrant further studies.

Figure 11. Immunofluorescence microscopy of adriamycin-treated primary mammary epithelial cells. Primary human mammary epithelial cells were treated for adriamycin 0, 500, and 1000 ng/ml for 6 hours. The cells were then analyzed by immunofluorescence microscopy for caspase-6 (green), lamin A/C (red), and DAPI (blue) in indirect immunofluorescence microscopy..

Note that as increase of adriamycin dosage, cytoplasmic caspase-6 translocates to nuclear, and nuclear lamin A/C became cytoplasmic.

BEC + Adriamycin (63×oil)



From the experiments of these tasks, we have made several very interesting observations. The observations that both transfection and expression of caspase-6 as well as adriamycin induced a very significant increase in lamin A/C protein level are very surprising and unexplained by the current literature. We may speculate that adriamycin induced nuclear localization and activation of caspase-6, and activated caspase-6 induces accumulation and cytoplasmic localization of lamin A. The findings imply that caspase-6 activity and localization may regulate lamin A/C dynamic: the protein level and localization. We hope to seek future support to continue the study.

Task 10 (month 12-14): Write final report for submission to DOD. Prepare manuscript to report the potential findings.

Milestone 4: We are able to conclude whether activated caspase-6 is present in breast cancer cells, and if the activation of caspase-6 and degradation of lamin A account for nuclear deformation and chromosomal instability in breast cancer.

The results of these pilot experiments support the initial hypothesis of a role of caspase-6 in lamin A/C degradation and cancer malignancy. Although, we recognize the complexity of the biology and relationship between caspase-6 and lamin A/C. The pilot study provides basis and promotes us to seek further investigation into the role of caspase-6 in cancer malignancy.

KEY RESEARCH ACCOMPLISHMENTS:

This research project has been completed and enabled us to obtain supportive information for the overall hypothesis that caspase-6 is increased in breast cancer and this may promote tumor cell invasion. The results are preliminary, though we are able to reach the following 3 conclusions.

1. We confirmed that caspase-6 expression and activity are commonly increased in malignant breast cancer tissues and cell lines.
2. We conclude that caspase-6 inhibition has some but limited influence in lamin A/C level in cancer cells.
3. We observed that p53 dependent induction or transfection of caspase-6 increase, rather than reduce, the cellular level of lamin A/C.

REPORTABLE OUTCOMES:

The concept award allowed us to make initial study of our hypothesis. The preliminary results confirm the increased expression of caspase-6 in breast cancer and support the hypothesis that the increased caspase-6 may degrade and regulate lamin A/C protein, and contribute to cancer development. The pilot results will be used to support application of additional fund to continue

With additional analysis, the increased expression of caspase-6 may be publishable. We will hope to further develop the data on cellular mechanistic analysis for future publication.

CONCLUSION:

The results of these pilot experiments confirm that caspase-6 expression is commonly increased in breast cancer tissues by immunostaining. We have also found that caspase-6 activity is commonly increased in breast cancer cells using an activity assay. In some but not all cells, we found that expression of caspase-6 correlates with the reduction of lamin A/C proteins. We also conclude that the relationship between caspase-6 and lamin A/C is more complex than we have initially recognized. Caspase-6 activation may degrade lamin A/C, but also has a positive impact on lamin A/C level.

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APPENDICES:

None

SUPPORTING DATA:

None