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PRINCIPAL INVESTIGATOR: Dr. Rafael Fridman

CONTRACTING ORGANIZATION: Wayne State University
Detroit, MI, 48202-4050

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Dr. Rafael Fridman
rfridman@med.wayne.edu

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14. ABSTRACT We report the progress of our studies focusing on the Discoidin Domain Receptors (DDRs), a set of kinase receptors that signal in response to collagen in prostate cancer. The project's goal is to define the expression and therapeutic potential of DDRs in prostate cancer. During the second funding period, we concluded the immunohistochemical study of DDR1 expression in a 200 case Grade/Stage tissue microarray (TMA) using a highly specific antibody. These studies described the localization of DDR1 in normal vs. tumor tissues and the association between staining intensity and Gleason score. We found that DDR1 expression may be an early indicator of PCa development. DDR1 is over-expressed in tumor tissues among the low Gleason score patients but not in the high Gleason score patients. We continued the studies to test a highly specific DDR1 blocking antibody (Ab) in intraosseous growth of human PC3 prostate cancer cells. This time we modified the protocol and found that pretreatment with the Ab diminished tumor incidence by bioluminescence. Thus, DDR1 may play a role in the initial seeding of tumor cells within the bone milieu. We are currently conducting the quantitative analyses of bioluminescence and the histomorphometry analyses and evaluation of effects on bone remodeling. Studies on DDR1 regulation and function in culture cells is ongoing.

15. SUBJECT TERMS
Prostate cancer, bone metastases, discoidin domain receptors, kinases, targeted therapies, immunohistochemistry

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1. INTRODUCTION

Subject: Treatment of prostate cancer (PCa) patients with bone metastases remains a challenge due to the limited arsenal of effective therapeutic drugs that reduce disease progression. Therefore, a major goal in PCa research is to identify specific targetable molecules to prevent and/or diminish the ability of PCa cells to survive within the intraosseous environment. The subject of our project is a set of receptor tyrosine kinases (RTKs), known as Discoidin Domain Receptors (DDRs), which signal in response to collagen, the major organic component of the bone extracellular matrix.
Purpose: To investigate the expression, therapeutic potential, and regulation of DDRs in PCa bone metastases.
Scope: Studies are proposed to define the expression of DDRs in PCa tissue specimens, the ability of DDRs to contribute to intraosseous tumor growth and define the regulation of DDRs in PCa cells.

2. KEYWORDS

Discoidin Domain Receptors, prostate cancer, bone metastases, collagen, tyrosine kinase, targeted therapy, extracellular matrix, signaling, antibodies,

3. ACCOMPLISHMENTS

- **What were the major goals of the project?**

Specific Aim 1. To investigate the expression of DDRs in our cohort of human PCa specimens and its association with clinical, pathological, and outcome data.

Task 1: To select and purchase tissue microarrays (TMA) from the Prostate Cancer Biorepository Network (PCBN).

Task 2: Conduct immunohistochemical (IHC) studies

Specific Aim 2. To evaluate the anti-cancer effects of DDR1 inhibitors in preclinical human-mouse xenograft models of primary and intraosseous PCa.

Task 3: Evaluate function-blocking antibodies in the orthotopic model of PCa

Task 4: Evaluate function-blocking antibodies in the intraosseous model of PCa

Specific Aim 3. To define the molecular and cellular bases of DDR regulation and signaling in PCa cell lines in cell based-assays.

Task 5: Analyses of DDR regulation, function, and signaling

- **What was accomplished under these goals?**

1) Major activities:

Task 1: We obtained a PCa tissue microarray (TMA) obtained from the Prostate Cancer Biorepository Network (PCBN) at the Johns Hopkins University Site. The TMA consist of 200 cases laid out in 5 slides containing 1600 core tissues. The TMA provides information on tumor stage and grade and is blinded in relation to patient identification, as required. An IRB (exempt) was approved by Wayne State University for the use of this TMA as requested by the PCBN and provided earlier to the CDMPR. This task is completed.

Task 2: The TMA was used for analyses of DDR1 by IHC using a DDR1 antibody that was obtained from Dr. Marco Prunotto (Roche). The antibody was evaluated for specificity and optimal concentration using normal and tumor tissues of various sources. We also tested for specificity using cell lines with or without DDR1 expression. Next, we stained the TMA using a protocol developed in our lab. The 1600 cores of the TMAs were evaluated by Dr. Dongping Shi, a pathologist with expertise in prostate cancer and co-investigator in this application. At the time of the previous report, we concluded the staining of the TMA and Dr. Shi, together with Drs. Fridman and Bonfil evaluated the intensity of the staining, the subcellular distribution (membranous, cytoplasmic and/or nuclear), and its association with Gleason score. During the period covered by this report (2016-2017), the data evaluated by Dr. Shi was analyzed for statistical significance by Dr. Wei Chen from the Biostatistics Core of the Karmanos Cancer Institute.

Task 4: During this period, we continue with the pre-clinical studies to evaluate the role of DDR1 in intraosseous tumor growth of the PCa cell line PC3 in mouse xenograft. We utilized two approaches to analyze the effect of DDR1: 1) Inhibition of DDR1 activity utilizing a highly specific neutralizing antibody referred to as RO6849889 antibody, obtained from Roche, and 2) Downregulation of DDR1 expression by shRNA. These studies were just completed and the data need to be evaluated.

Task 5: We investigated the effects of the blocking anti-DDR1 Ab on various cellular activities in PC3 cells. Studies were also conducted to establish the subcellular localization of DDR1 in PCa cell lines.

2) Specific objectives:

The objectives during the period cover by this report were:

a. Complete the evaluation of DDR1 expression in the PCa TMA by performing the statistical analyses to determine the association of DDR1 levels and subcellular localization with disease progression.

b. Investigate the role of DDR1 in intraosseous growth of PC3 cells.

c. Investigate the role and subcellular distribution of DDR1 in PCa cells.

3) Significant results or key outcomes:

Task 1. Completed.

Positive Outcomes: The TMA was obtained.

Negative Outcomes: None to report.

Task 2: We completed the analyses of DDR1 expression in the PCa TMA. Specifically, the TMA consisted of paired and repeated tumor and normal specimens from each of 200 PCa patients (**Table 1**). For each specimen, positive or negative IHC staining was evaluated. For each patient, staining intensity was defined as percentage of positive or negative staining on repeated specimens of same type, within tumor tissues or normal tissues separately, TMA specimens that had stroma, no glands, or no tissue after staining process will be considered as missing at random rather than negative staining. Three cellular locations, including membrane, nuclear, and cytoplasm, were assessed separately (**Table 2**). Patient baseline characteristics, such as Gleason scores and TNM stage, were reported descriptively. Categorical data are reported as frequencies and percentages. Univariate analysis of association between Gleason scores (3+4 or lower vs 4+3 or higher) and dichotomized staining intensities (0% vs >0%) within each tissue type and each cellular location was performed using Fisher's exact test (**Fig. 1**). The differential expression of paired tissue types was categorized into no difference (0%), over-expression (>0%), and under-expression (<0%). The pattern of the differential expressions was then compared between low grade and high-grade Gleason groups using Fisher's exact test (**Fig. 2**). The association between continuous staining intensities and high/low Gleason grades was also evaluated using multivariable logistic regression adjusted for TNM stage. All p values are 2-sided with a significance level of .05. The results of these analyses should be regarded only as descriptive findings and multiple testing were not adjusted. All calculations were performed with R version 3.0.2.

Table 1. Patient characteristics

Variable	Frequency (n=200)
Gleason Score	
Low grade (≤(3,4))	166 (83%)
High grade (≥(4,3))	34 (17%)
TNM Stage*	
Local	132 (66%)
Advanced	68 (34%)

*: metastasis was not assessed and there was no T1 in this dataset. Local: T2N0; Advanced: any T and N1 or higher.

Table 2. DDR1 Staining results by subcellular location and tissue type

	Normal (n=887)‡	Tumor (n=713)
Membranous Staining*		
No Staining	19 (2%)	9 (1%)
Basal Only	214 (24%)	90 (13%)
Basal-Lateral	470 (53%)	316 (44%)
Full Membranous	76 (9%)	211 (30%)
Stroma/No Glands	74 (8%)	43 (6%)
No tissue	34 (4%)	44 (6%)
Nuclear Staining		
No Staining	765 (86%)	590 (83%)
Positive	17 (2%)	36 (5%)
Stroma/No Glands	72 (8%)	43 (6%)
No tissue	33 (4%)	44 (6%)
Cytoplasmic Staining		
No Staining	763 (86%)	557 (78%)
Positive	19 (2%)	69 (10%)
Stroma/No Glands	72 (8%)	43 (6%)
No tissue	33 (4%)	44 (6%)

‡All tissue spots (normal or tumor) were re-evaluated by Dr. Shi. Some tissue spots, which were tumor by TMA design, were re-categorized into normal after pathological evaluation by Dr. Shi.
*Basal only or basal-lateral was grouped with no staining as negative staining results.

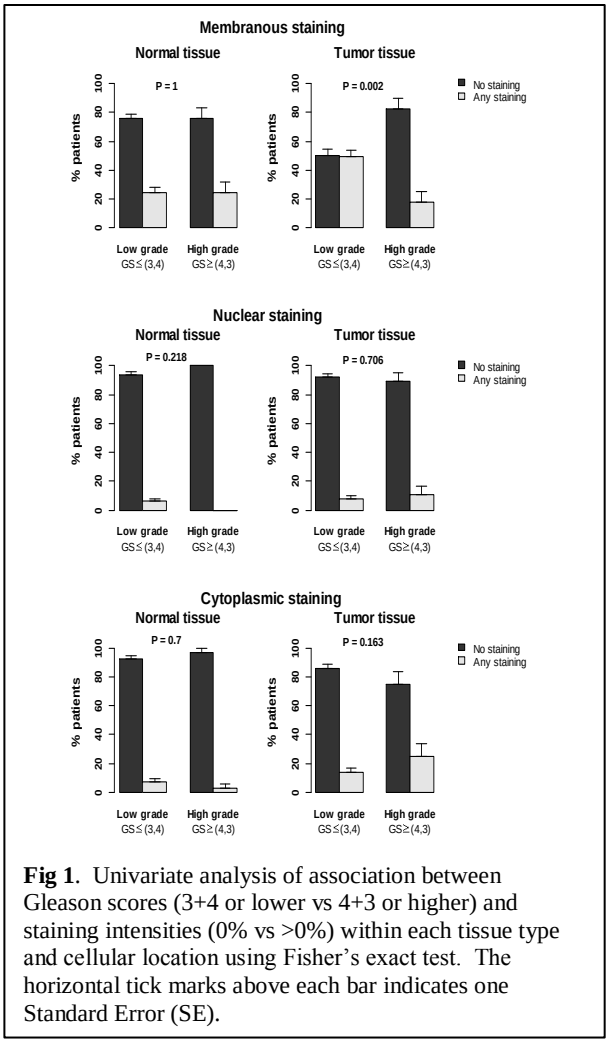

Fig 1. Univariate analysis of association between Gleason scores (3+4 or lower vs 4+3 or higher) and staining intensities (0% vs >0%) within each tissue type and cellular location using Fisher's exact test. The horizontal tick marks above each bar indicates one Standard Error (SE).

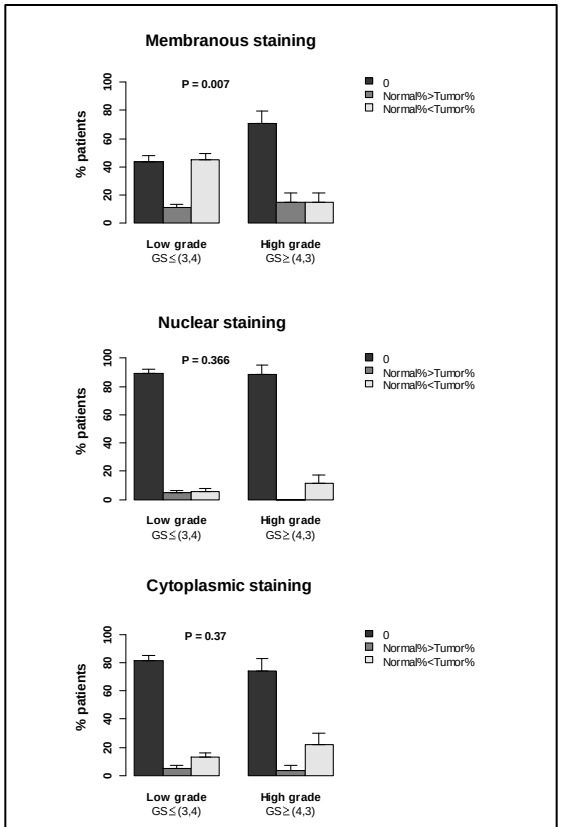

Fig. 2. The differentiation of paired tissue types was categorized into no difference, over-expression (normal% < tumor%), and under-expression (normal% > Tumor%.) The pattern of the differentiation was then tested between low-grade and high-grade groups using Fisher's exact test.

Table 3. Logistic regression of high/low Gleason grade of DDR1 staining intensity in cell membranes

	Tumor tissues		**Normal tissues**		**Paired Tumor/Normal***	
	OR (95% CI)	P value	OR (95% CI)	P value	OR (95% CI)	P value
Staining Intensity	0.97 (0.95,0.99)	0.001	1.00 (0.98, 1.03)	0.727	0.97 (0.96,0.99)	0.001
TNM stage (ref: local)	8.82 (3.39,22.94)	<0.001	11.61 (4.65,28.96)	<0.001	11.44 (4.16, 31.48)	<0.001

* Staining intensity difference (tumor % - normal %)

Note: Nuclear and cytoplasmic staining intensities were not significant.

Positive Outcomes: DDR1 is expressed mostly in membrane of the epithelial cells. No significant expression was detected in stromal cells. Based on the intensity of the staining, the data suggest that DDR1 could be an early cursor of PCa. DDR1 is over-expressed in tumor tissues among the low Gleason score patients. This over-expression was not observed among the high Gleason score patients.

Negative Outcomes: None to report. However, we do not possess survival data o disease progression. We have also no correlation with presence of bone metastases. We hope to get more information from the source of the TMA (PCNB).

Task 3: Nothing to report at this stage. We have not yet started the experiments aimed at examining the role of DDRs in the orthotopic model of PCa. The reason we have delayed this experiment was to focus first on the intraosseous model and determine whether we observe a therapeutic effect and learn about this new DDR1 inhibitor. We are planning to conduct these studies in the upcoming funding period.

Task 4. In the previous report, we demonstrated the ability of the RO6849889 antibody to inhibit DDR1 collagen-induced activation in PC3M-Luc2 cells, which promoted us to test its effects on the intratibial model of bone metastases using PC3M-Luc2 cells. We reported that the results of that study were inconclusive due to: 1. The aggressiveness of the cells, which caused bone fractures, and thus made it difficult to conduct histomorphometry analyses to measure tumor burden within the bone. 2. We need to improve the use of bioluminescence (BLI) to better evaluate tumor burden. 3. Antibody schedule. Although the antibody is quite stable in mouse, we thought that it will be better to re-evaluate schedule. These lessons were utilized to modified the original protocol. During the period of this reporting, we performed two major studies in mice. The first experiment followed the protocol of the experiment described in the previous report in which $5x10^5$ PC3M-Luc2 cells were inoculated intratibially and antibody administration was given on days 7, 14, and 21 days after tumor cell inoculation. As mentioned above, the first experiment, although it suggested a small therapeutic effect of the Ab based on BLI, it was inconclusive because we couldn't perform rigorous histomorphometry analyses due to the presence of multiple tibial fractures that disrupted the continuity of the tumor tissue. Thus, tumor burden under the various conditions couldn't be determined. We hypothesized that the excess of factures was due to the relatively high number of tumor cells inoculated. Therefore, we conducted a new experiment in which we reduced the number of tumor cells in half. In addition, we changed the time of administration of the blocking antibody and the control. In one group, the compounds were administered at Day 2 after tumor cell inoculation, as opposed to

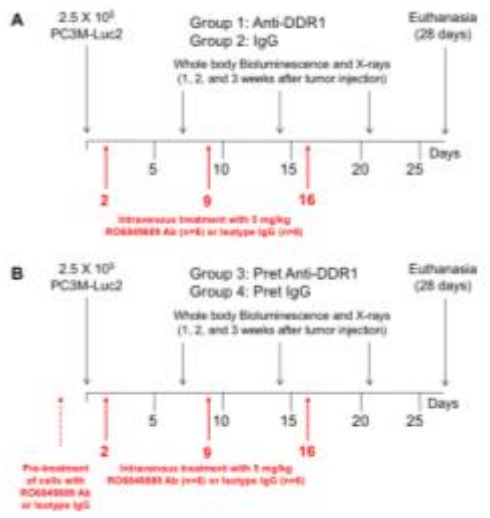

Fig. 3. Treatment protocols to target DDR1 in mice harboring intratibially injected PC3M-Luc2 cells. We used six mice per group (n = 6). Mice were euthanized 4 weeks after from tumor cell inoculation.

Day 7. We postulated that waiting 7 days to administer Ab would not be efficient due to tumor burden. Thus, Ab administration was initiated at day 2 after tumor cell inoculation. Another experimental group consisted of mice inoculated with tumors cells that were pre-incubated with the compounds before inoculation. We postulated that this approach will inhibit DDR1 activity at the time of seeding and possibly decrease tumor implantation and subsequent growth within the bone. **Fig. 3** depicts the two main experimental groups of the study conducted during this funding period. Panel A depicts the protocol of the groups in which treatment (with anti-DDR1 Ab or IgG) was initiated two days after tumor cell inoculation. Panel B depicts the protocol for the groups of mice in which treatments were initiated *ex-vivo* by incubating (60 min at 4ºC) PC3M-Luc2 cells with the anti-DDR1 Ab or the control IgG. We have confirmed that this timeframe of antibody treatment, inhibits collagen-evoked DDR1 activation (data not shown). This group also received the first treatment two days after inoculation. Whole body BLI and X-rays were performed at week 1, 2 and 3 after tumor injection. Mice were euthanized at day 24, their tibiae were harvested and subjected to *ex vivo* X-ray imaging using the Trident Digital Specimen Radiography system. The X-ray images were used to determine bone response (osteolytic, osteosclerotic or mixed) in untreated or treated mice.

Positive Outcomes: BLI was detected, indicative of tumor implantation and growth. Moreover, BLI, *ex vivo* X-rays, panCK IHC and H&E analyses demonstrated that mice in groups 1 and 2 displayed 100% intraosseous tumor incidence. Group 3 showed 60% (or 80% if the % tumor area of 0.58 found in tissue sections is considered), as opposed to 100% tumor incidence in Group 4. **Fig. 4** shows the BLI after 3 weeks of tumor cell inoculation in the four experimental groups. We also obtained tissue sections for histomorphometry analyses, which are currently being conducted. We are also quantifying the BLI. Regardless, the preliminary data suggest that pre-treatment of the cells with the blocking antibody may reduce tumor incidence/uptake. This is suggested by the lack of BLI in 2/5 mice of Group 3 vs. Group 4 in which 6/6 mice show BLI (Fig. 4). Although preliminary, these are very encouraging results. **Negative Outcomes:** The mice of Group 1, treated with the antibody after two days, showed no apparent visual differences in BLI when compared to the control mice of Group 2 (Fig. 4). We had some difficulties in mastering the procedure of BLI measurements. Therefore, quantitative data of BLI are not available at the time of this writing. We are working to solve this problem. We would also like to know whether the antibody is working in the tumor cells within the bone. For this, we will plan to utilize the antibodies that recognize phosphorylated DDR1 and develop a protocol for IHC.

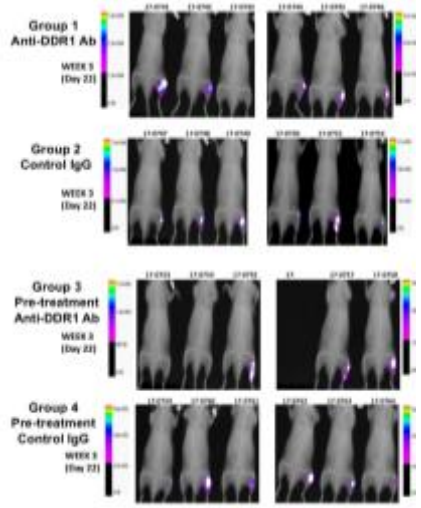

Fig. 4. BLI images at week 3 after tumor cell inoculation. Please note, in group 3, one mouse died at week 1.

In addition to quantifying BLI and conducting histomorphometry analyses, we are also analyzing the tissue sections for bone remodeling and other histopathological markers (cell proliferation, apoptosis, etc.). Therefore, a final conclusion from this experiment awaits the results of those

analyses. For an alternate approach to evaluate the role of DDR1 in intraosseous tumor growth, we have generated PC3 cells with downregulated DDR1 expression via shRNA.

Task 5: Nothing to report at this time. These studies are ongoing and the data are still too preliminary. However, we have conducted the following experiments: Roles of DDR1 in proliferation and invasion. Studies on the subcellular distribution of DDR1. Studies of the effect of DDR1 on regulation of osteolytic factors.

4) Other achievements.

Nothing to Report.

- **What opportunities for training and professional development has the project provided?**

Nothing to Report

- **How were the results disseminated to communities of interest?**

Nothing to Report

- **What do you plan to do during the next reporting period to accomplish the goals?**

For the next reporting period, we are planning the following studies based on the SOW:

Tasks 1 and 2: To conclude this study, we are currently taking photographs of the various sections of the TMA to represent the findings. We plan to evaluate antibodies for DDR2, to examine its expression in PCa specimens. We tested few antibodies but still we are not convinced of the specificity. We are working the conditions for IHC. In addition, we plan to test anti-DDR1 antibodies that recognize the phosphorylated receptor in IHC. If successful, these analyses will provide important information on the status of receptor activation within the tissues. Finally, we plan to conduct IHC in sections of primary tumor and bone metastases.

Task 3: As stated above, we plan to initiate these studies using PC3M-Luc2 cells. We are planning first to inoculate a few mice to determine the optimal cell number and time of progression. Then we will design the treatment experiment.

Task 4: We will continue with the analyses of the tissues obtained in the intratibial model. Based on these results, we may consider to conduct another study with the pre-treatment conditions, which so far looks promising.

Task 5: We are conducting the studies of this Task, aimed at analyzing the roles of DDR in culture systems.

4. IMPACT

- **What was the impact on the development of the principal discipline(s) of the project?**

Nothing to Report.

- **What was the impact on other disciplines?**

Nothing to Report.

- **What was the impact on technology transfer?**

Nothing to Report.

- **What was the impact on society beyond science and technology?**

Nothing to Report.

5. CHANGES/PROBLEMS

- **Changes in approach and reasons for change**

There are no changes in the approach.

- **Actual or anticipated problems or delays and actions or plans to resolve them**

We do not anticipate major technical problem that cannot be resolved or tackled with a different approach. The experiments using the orthotopic model have not been initiated yet but these are challenging because it requires animal surgery. We will carefully plan those studies and request assistance from the Animal Core at the Karmanos Cancer Institute, if necessary.

- **Changes that had a significant impact on expenditures**

No changes in the period of this report.

- **Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents**

There were no changes in use or care of human subjects, vertebrate animals, and/or select agents. We do not anticipate future changes in these categories for the upcoming funding period.

6. PRODUCTS:

- **Publications, conference papers, and presentations**

Nothing to Report

- **Website(s) or other Internet site(s)**

Nothing to Report

- **Technologies or techniques**

Nothing to Report

- **Inventions, patent applications, and/or licenses**

Nothing to Report

- **Other Products**

Nothing to Report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

- **What individuals have worked on the project?**

Name	Project Role	Nearest Person Months Worked	Contribution to Project	Funding Support
Rafael Fridman	PI	0.48	Design of experiments data analyses	This grant
Daniel Bonfil	Co-PI	0.48	Design of experiments data analyses	This grant
Dongping Shi	Co-PI	0.12	Analyses of TMA	This grant
Wei Chen	Biostatistician	0.12	Statistical analyses	This grant
Allen Saliganan	Research Scientist	5.40	Animal studies, immunostaining	This grant
Anjum Sohail	Research Scientist	5.40	Studies in cell culture	This grant
Benjamin Wasinski	Research Assistant	9	Studies in cell culture	This grant

- **Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?**

No changes in Other Support to report for this period for the PI or any other senior/key personnel.

However, PLEASE NOTE: Dr. Bonfil, co-PI in this award, resigned from his faculty position at Wayne State University on July 28, 2017. We reported this change to the DoD in the middle of July, requesting to change the position of Dr. Bonfil to Paid Consultant and provided all the requested information. On July 27, 2017, we received an e-mail from Ms. Jennifer Shankle that our request was approved. Thus, beginning on August 1, 2017, Dr. Bonfil is a Paid Consultant.

- **What other organizations were involved as partners?**
    - **Organization Name:** Hoffmann-La Roche
    - **Location of Organization:** Basel, Switzerland
    - **Partner's contribution to the projec**t
        - **Other:** Supplied the neutralizing antibody to DDR1, referred to as RO6849889.

8. SPECIAL REPORTING REQUIREMENTS

Nothing to Report

9. APPENDICES:

Nothing to Report