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**14. ABSTRACT**

T2D frequently occurs together with obesity. In fact, the vast majorities of T2D individuals, both civilians and Veterans are or have been overweight or obese. Surprisingly, the majority of the obese subjects, Veterans or non-veterans, never develop T2D and their metabolic tissues will never lose the ability to respond to insulin. This fact indicates the critical role of the genetic predisposition to T2D development in obesity. The proposal assesses for the first time if pathogenic single nucleotide variations (SNVs) within three genes, i.e., *PIKFYVE*, *FIG4* and/or *VAC14*, found previously to be involved in glucose homeostasis, are linked with T2D development in obese individuals. The expected changes will be related to functional differences in the performance of the PIKfyve enzymatic activity. A total of 96 obese patients with or without T2D (DM and non-DM), 48 per group, with similar average BMI and age have been selected and included in the analyses. Fat tissue for analyses has been collected and DNA was isolated. Samples were subjected to next-generation exome sequencing that was followed by comprehensive bioinformatics analysis. Several promising SNVs in the three genes, with $p<0.05$, associated specifically with T2D in the obese individuals have been already identified. Determination of their position revealed that all of the T2D-asssociated SNPs are located in the noncoding regions of the three genes. Among those, of particular interest are T2D-associated super enhancer SNVs identified in the *PIKFYVE* gene of fat tissue. We are currently extending our observations by comparing our findings with those available in database or by other research groups to gain novel insights in the pathogeneses of T2D in obesity.

**15. SUBJECT TERMS**
type 2 diabetes mellitus; morbid obesity; PIKFYVE, FIG4 and VAC14 genes; single nucleotide variations

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1. **INTRODUCTION:** Patients suffering from Type 2 Diabetes mellitus (T2D) are usually overweight or obese. However, only a small fraction of even morbidly obese individuals develops T2D. Since T2D is a familial disease, a genetic predisposition for the development of T2D is expected to play a major role in obese individuals. Basic studies have shown that manipulating the levels of 3 cellular proteins -PIKfyve (encoded by *PIKFYVE* gene), Sac3 (encoded by *FIG4* gene) and ArPIKfyve (encoded by *VAC14* gene) – which associate in a functional complex, markedly affects insulin sensitivity of fat cells. Furthermore, knockout of mouse *pikfyve* selectively in muscle or fat cells causes whole body insulin resistance in transgenic mice. Therefore, it is plausible that mutations in the three genes of interest could cause tissue insulin resistance and thereby predispose the obese individuals to develop T2D.

2. **KEYWORDS:** T2D (type 2 diabetes mellitus); morbid obesity; PIKFYVE, FIG4 and VAC14 genes; single nucleotide variations

3. **ACCOMPLISHMENTS:** It should be mentioned that there was misunderstanding related to the start day of this award. Whereas our University official and myself were informed by DoD that the start day will be June 15, 2017 as we requested, in reality the start day given by DoD was April 15, 2017. The later start day was requested based on the delayed decision by the our University IRB committee. Therefore, we requested a 1-year no-cost extension that was granted to us by the DoD officials on September 26, 2018. The grant duration in this case is until October 14, 2019.

The proposed goals of the project for the first year are completed according to our SOW. Namely the goals for the first year as appeared in our SOW (Table 1) were:

Table 1: Report of work: start day: **June 15/2017 – October 14/2018**

| **Specific Aim 1: Identify non-synonymous single nucleotide variations (SNVs) within *PIKFYVE*, *FIG4* or *VAC14* genes specifically associated with T2D in obesity by exome sequencing:** | **Timeline Months (-)1-7** | Accomplished |
|---|---|---|
| **Major Task 1 Request for approval for research with Human Anatomical Substances and assembly of the two groups** | | |
| Subtask 1: U.S. Army Medical Research and Materiel Command (USAMRMC) Office of Research Protections (ORP), Human Research Protection Office (HRPO) approval prior to research implementation for the entire study | (-)1-2 | Accomplished |
| Subtask 2: Local IRB/EC Approval for the entire study | (-)1-2 | Accomplished |
| Subtask 3: Evaluate and Divide participants to two groups: Obese with T2D and Obese without T2D | 3 | Accomplished |
| **Major Task 2** | | |

| | | |
|---|---|---|
| **Isolate genomic DNA and perform whole exome sequencing** | | |
| Subtask 1: Isolate genomic DNA | 4-5 | Accomplished |
| Subtask 2: Exome sequencing on fee-for-service basis by next generation sequencing technology at the Wayne State University Core for Applied Genomics Technology Center (AGTC). I have already consulted with Dr. Susan Land, Director of the AGTC Core, who has agreed to accept and prioritize our project. | 6 | Accomplished |
| Subtask 3: Perform bioinformatics consulting with bioinformatician about the computational predictions from all available web servers. | 7 | Accomplished |
| Milestone(s) Achieved: Identified are coding SNVs within the three genes associated with T2D in obesity | 7 | Accomplished |
| **Specific Aim 2: Establish T2D-associated coding SNVs in *PIKFYVE*, *FIG4* and/or *VAC14* in obesity, leading to quantitative differences in synthesis of PI(3,5)P2 and/or PI5P** | Months 8-18 | |
| **Major Task 3**<br>**Prioritize SNVs for analyses** | | |
| Subtask 1: Analyze the coding SNVs relative to its localization in the protein itself and in the triple protein complex using the interaction map. Prioritize SNVs | 8 | Accomplished |
| Subtask 2: Select fat tissue with the prioritized SNVs | 9 | Accomplished |
| Milestone(s) Achieved: Selected are ~50 priority T2D-causing SNVs in the three proteins | 9 | Accomplished |
| We need to repeat the sequencing of 5 sample | | In process |
| Subtask 3: Perform functional assays of the lipid kinase activity and analyze proteins levels by WB simultaneously in order to thaw tissue only once. | 9-13 | In process |
| Subtask 4: Mutagenesis to introduce the variations in the cDNA and cell transfections | 14-16 | Will not be required because no SNVs in the coding region were found |
| Subtask 5: Relate changes in protein levels with the data from the functional assay | 17 | In process |
| Milestone(s) Achieved: Design a map with T2D-causing SNVs in the three proteins that alter synthesis of PI(3,5)P2 and PI5P | 18 | In process |
| Subtask 7: Deposit the identified T2D-causing SNVs in the three proteins that alter synthesis of PI(3,5)P2 and PI5P in the related webservers | 18 | In process |

- ***What was accomplished under the above goals:***

| Female | | | | Male | | | |
|---|---|---|---|---|---|---|---|
| Obese non-Diabetic | | Obese Diabetic | | Obese non-Diabetic | | Obese Diabetic | |
| 32 | | 32 | | 16 | | 16 | |
| AA | W | AA | W | AA | W | AA | W |
| 21 | 11 | 21 | 11 | 10 | 6 | 10 | 6 |

Table 2: Selected individuals for the study

***1)*** As proposed, ninety six (96) morbidly obese individuals with or without T2D were selected and were matched by gender and race [AA (African American), W (White)] as shown in the Table 2 below:

The age between the obese diabetic group (a total of 48) and obese non-diabetic group (a total of 48) were not statistically different (Table 3)

| | Obese non-Diabetic | Obese Diabetic |
|---|---|---|
| Total | 48 | 48 |
| Age | mean = 42.0 $\pm$ 2.85 | mean = 47.0 $\pm$ 1.5 |
| BMI | mean = 49.4 $\pm$ 1.06 | mean = 47.9 $\pm$ 1.01 |

Table 3: Age and BMI of the groups

***2)*** As proposed, the frozen samples of abdominal fat from these individuals, collected during laparoscopic bariatric surgery of morbidly obese individuals were successfully used for genomic DNA extraction and next generation exome sequencing of the genes of interest. As proposed, we used fee-for-service Illumina next-generation sequencing housed by the AGTC/Karmanos Genomics Core for the Cancer center located in our Institution.

***3)*** The result of the exome sequences came back to us about 2 months ago. We focused on the sequences of the 3 genes of interest, namely *PIKFYVE*, *VAC14* and *FIG4* as proposed. In those three, we identified numerous (>400) SNVs. However, our bioinformatics analyses indicated that the facility did not do a good job with 5 of our samples in the group of Obese Diabetics. These 5 samples will be re-sequenced.

***4)*** We subjected the SNVs to analysis for the association between polymorphisms and T2D using a logistic regression model, by adjusting age, gender and BMI. Associations were examined in each of the two populations separately.

***5)*** So far, our bioinformatics analysis indicated that in the African American (AA) population, a total of 13 SNVs identified in the three gene sequences were significantly ($p<0.05$) or marginally ($p<0.1$) associated with T2D. In the Caucasian population, as many as 30 polymorphisms were identified in the exome sequencing with statistical ($p<0.05$) or marginal ($p<0.1$) significance to be associated T2D.

***6)*** 1 - performed a quality control test to identify that 5 samples were not sequence at the expected high level. Will be repeated n the expense of the sequencing core.

- ***What do you plan to do during the next reporting period and beyond***

We will continue with our tasks as specified in the Table of Statement of work. Specifically:

1 - We will perform a quality control with a HWE (Hardy-Weinberg equilibrium) test;

2 - We will further validate the established polymorphisms by using independent genotyping techniques;

3 - We will progress with the in vitro analyses of the PIKfyve product PtdIns(3,5)$P_2$ and PtdIns5P of the samples that indicate statistically significant SNP. This will allow us to make an association between polymorphisms and enzymatic activities.

4 – Thus far, all our SNVs are in the non-coding sequence of all 3 genes. Therefore, mutagenesis as previously proposed (within Specific Aim 2) appears to not be necessary.

5 - It appeared that the core facility that performed the exome sequencing had a failure with 5 of our samples. To remedy this mishap the facility offered to re-sequence the 5 samples for free. However, the offer was contingent upon the condition that our 5 samples will be included in the sequencing of another client. So, we have to wait for the sequencing data of the 5 sample to come and in order to further evaluate data by bioinformatics.

4. **IMPACT:** The preliminary analysis supports the hypothesis that single nucleotide variations in the genes of interest are associated with T2D in certain morbidly obese patients. At this point it is early to determine the impact on other disciplines, on technology transfer and on society. However, identification of the T2D-associated SNVs in the super enhancer region of PIKfyve in the African-American group is very promising. We are now waiting for the re-sequencing of the 5 samples to identify whether these SNVs are race dependent.

5. **CHANGES/PROBLEMS:** Our request for one-year no-cost extension has been granted. Unfortunately, we have to wait for further data from the sequencing of the 5 samples in order to complete the proposed project. The 5 samples were not sequence at the expected quality due to a failure of the sequencing facility.

6. **PRODUCTS**: An Abstract has been submitted in the 2018 Annual Meeting of the American Diabetes Association but unfortunately has not been accepted for presentation due to the preliminary nature of our study. While we were waiting for sequencing data, we published 2 papers, supported partly by DoD, to further characterize the pathology of PIKfyve dysfunction. We were able to finish more work because mutagenesis was not necessary step, given that all of the SNVs

were located in the non-coding region of the 3 genes. In case a better quality sequencing with the 5 samples identify SNVs in the coding regions, we will continue with mutagenesis should fund from other sources be available.

1. Ikonomov, OC, Altankov, G, Sbrissa, D, **Shisheva, A**: PIKfyve inhibitor cytotoxicity requires AKT suppression and excessive cytoplasmic vacuolation; Toxicol Appl Pharmacol. 2018 Oct 1;356:151-158. doi: 10.1016/j.taap.2018.08.001. Epub 2018 Aug 9.

2. Sbrissa, D, Naisan, G, Ikonomov, OC, **Shisheva, A:** Apilimod, a candidate anticancer therapeutic, arrests not only PtdIns(3,5)P2 but also PtdIns5P synthesis by PIKfyve and induces bafilomycin A1-reversible aberrant endomembrane dilation. PLoS One. 2018 Sep 21;13(9):e0204532. doi: 10.1371/journal.pone.0204532. eCollection 2018.

7. **PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS: The following individuals**

Note, during our 1-year no-cost extension, only the principal investigator will work on this grant under 0.6% effort.

| | |
|---|---|
| Name, ID | Assia Shisheva |
| Role | Principal Investigator |
| Contributions | Managing all activities |
| Nearest Month worked | 0.6 |
| Funding support | none |

8. **SPECIAL REPORTING REQUIREMENTS**: Note, during our 1-year no-cost extension, only the principal investigator will work on this grant under 0.6% effort. All of the other collaborators are off the grant due to my 4.5 months sabbatical as well as lack of funds.

9. **APPENDICES:** None