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Fusion Genes Predict Prostate Cancer Recurrence

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CONTRACTING ORGANIZATION:
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13. SUPPLEMENTARY NOTES

14. ABSTRACT

Most prostate cancers are indolent, and may not require clinical intervention. Only a small fraction of cases progress to metastatic disease if not treated. Prediction of the clinical outcomes of prostate cancer remains a challenge. Recently, we discovered a panel of 8 fusion genes that occurred in aggressive prostate cancer. The presence of these fusion genes correlates 91% chance of recurrence of prostate cancer. In order to make the fusion gene test clinically ready as a predictor, we have modified to test into a semi-quantitative Taqman QRT-PCR. The tests were performed in CLIA certified lab. Four hundred and sixty samples of prostate cancers were collected. Prostate cancer cells were microdissected. Taqman QRT-PCRs were performed on these samples. Significant numbers of samples were found positive for some of these fusion genes. In addition, we have analyzed 150 samples from Stanford University and 155 samples from University of Wisconsin Madison. A training model has been constructed. We are in the process to apply the model to testing sets.

15. SUBJECT TERMS
None listed.

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1. INTRODUCTION:

Cancer specific fusion genes are the results of chromosome rearrangement in the cancer genomes. The detection of fusion transcripts in cancer cells may reflect the progression of human cancer. Previously, we have identified a panel of 8 fusion genes in prostate cancer. The presence of these fusion transcripts correlated with the aggressive behavior of prostate cancer. In this proposed study, we will conduct large scale analysis to evaluate whether the detection of these fusion transcripts is predictable for poor clinical outcomes.

2. KEYWORDS:

Fusion gene, RNA, Taqman RT-PCR, in situ hybridization, RNA, prostate cancer, cancer relapse, chromosome

3. ACCOMPLISHMENTS:

What were the major goals of the project?

We will conduct analysis of MAN2A1-FER, SLC45A2-AMACR, TRMT11-GRIK2, MTOR-TP53BP1, LRRC59-FLJ60017, CCNH-C5orf30, KDM4-AC011523.2, TMEM135-CCDC67 on over 1000 prostate cancer samples collected from University of Pittsburgh, Stanford University and University of Wisconsin Madison. We will first establish prostate cancer recurrence model and short PSADT prediction models either by fusion gene status alone or in combination with nomogram based on the cohort from 600 radical prostatectomy samples from UPMC. This model will be locked in and tested on cohorts from University of Pittsburgh, Stanford University and University of Wisconsin. The prediction accuracy, sensitivity and specificity within each cohort will be evaluated.

1) In the first 3 months of the funded period, we plan to establish this test in the CLIA certified laboratory at the University of Pittsburgh Medical Center. Fifty-six FFPE samples that were shown to be positive for at least one fusion transcripts in the matched frozen tissues. Detection threshold will be obtained.

2) From month 4-9 of the first funded year, we will perform TAQMAN QRT-PCR and Sanger's sequencing on a randomly selected cohort of 600 samples from phase 1 that have at least 5 years clinical follow-up. These tests will be performed in CLIA certified laboratory of University of Pittsburgh. The prediction models of PCa recurrence and PSADT mentioned will be developed based on this large number of samples. For sites 2 and 3, the first 300 prostate cancer cases from each site will be selected and evaluated for sufficient materials for the assay.

3) TAQMAN QRT-PCR analysis for the fusion genes will be carried out at the CLIA certified lab at the University of Pittsburgh using approximately 200 samples provided by Drs. Brooks and Jarrard. In addition, validation of selected prostate cancer samples on specific fusion genes using FISH will be performed. Statistical analyses will be performed to evaluate whether the fusion gene status is predicative for the clinical outcomes of prostate cancers.

What was accomplished under these goals?

Major Task 1 : We will conduct analysis of MAN2A1-FER, SLC45A2-AMACR, TRMT11-GRIK2, MTOR-TP53BP1, LRRC59-FLJ60017, CCNH-C5orf30, KDM4-AC011523.2, TMEM135-CCDC67 on 5106 prostate cancer samples collected from University of Pittsburgh, Stanford University and University of Wisconsin Madison. We will first establish prostate cancer recurrence model and short PSADT prediction models either by fusion gene status alone or in combination with nomogram based on the cohort from 600 radical prostatectomy samples from UPMC. This model will be locked in and tested on cohorts from University of Pittsburgh, Stanford University and University of Wisconsin. The prediction accuracy, sensitivity and specificity within each cohort will be evaluated.

Subtask 1: In the first 3 months of the funded period, we plan to establish this test in the CLIA certified laboratory at the University of Pittsburgh Medical Center. Fifty-six FFPE samples that were shown to be positive for at least one fusion transcripts in the matched frozen tissues. These FFPE samples had been tested in non CLIA certified laboratory, and achieved 98.9% sensitivity and 100% specificity. We will repeat the same tests on these samples in CLIA certified laboratories. All PCR products will be analyzed through Sanger's sequencing to confirm the authenticity of the fusion products. In addition, all fusion minigene RNA templates will be serially diluted. TAQMAN QRT-PCR will be performed to evaluate the sensitivity of the test. Detection threshold will be obtained. Random selection of 600 prostate cancer samples with definitive clinical outcomes will be carried out in UPMC campus. TAQMAN QRT-PCR on β-actin will be used as RNA quality control. For sites 2 and 3, all relevant institutional review board exempt protocols will be secured and approved.

Progress: We have procured the CLIA certified lab space in the beginning of the funded period. To accommodate the reality of formalin-fixed and paraffin-embedded tissues, we have designed a set of new primers and Taqman PCR probes for highly fragmented RNA species. These sets of primers and probes were subsequently tested and validated on synthetic mini-fusion genes of MAN2A1-FER, TRMT11-GRIK2, MTOR-TP53BP1, CCNH-C5orf30, KDM4-AC011523.2, SLC45A2-AMACR, TMEM135-CCDC67, and LRRC59-FLJ60017. The probe and primers for β-actin were also revised to accommodate a shorter RNA fragment. The analyses showed that these assays detect as low as 600-1000 molecules of these fusion transcripts. We then analyzed 56 FFPE samples whose frozen counterparts have been previous found to contain at least one fusion gene using these sets of probes and primers. All samples that were positive for these fusion genes were also positive in the new Taqman qRT-PCR assays. The positive match rate is 100%. All participating institutes, including University of Pittsburgh, Stanford University and University of Wisconsin Madison, had obtained the institutional approval for the exempt protocols.

Subtask 2: From month 4-9 of the first funded year, we will perform TAQMAN QRT-PCR and Sanger's sequencing on a randomly selected cohort of 600 samples from phase 1 that have at least 5 years clinical follow-up. These tests will be performed in CLIA certified laboratory of University of Pittsburgh. The prediction models of PCa recurrence and PSADT mentioned will be developed based on this large number of samples. For sites 2 and 3, the first 300 prostate cancer cases from each site will be selected and evaluated for sufficient materials for the assay.

Progress: To create a training set, we performed Taqman qRT-PCR using the primers and probes as mentioned from above on 271 samples from University of Pittsburgh, 155 samples from University of Wisconsin Madison, and 150 samples from Stanford University. The results show surprisingly high positive rate of SLC45A2-AMACR in Stanford and Wisconsin cohort, reaching 96% and 92.6% respectively. Among these fusion genes, the lowest frequent

Table 1 Positive rate of fusion in prostate cancers

Cohort	MAN2A1/ FER	TRMT11/ GRIK2	MTOR/ TP53BP1	CCNH/ C5orf30	KDM4B/ AC011523.2	SLC45A2/ AMACR	TMEM135/ CCDC67	LRRC59/ FLJ60017
UPMC	13% (60)	25.8% (119)	2.8% (13)	33.4% (154)	0.4% (2)	50.1% (234)	1% (5)	3.4% (16)
Stanford	18% (9)	20% (10)	10% (5)	12% (6)	4% (2)	96% (48)	6% (3)	22% (11)
UWisc	19% (31)	12.9% (21)	4.3% (7)	76.7% (125)	9.2% (15)	92.6% (151)	0.6% (1)	26% (43)

Table 2
The cutoffs(and OR) of each fusion gene in each cohort

Cohort	MAN2A1/FER	MAN2A1/FER-actin	TRMT11/GRK2	MTOR/TP53BP1	CCNH/C5Orf30	KDM4/AC011523.2	SL45A2/AMACR	TMEM135/CCDC67
UPMC	32(26.3)	0 (25.9)	43(5.53)	42(inf)	39(0.12)	44(inf)	34(1.57)	47(1.54)
Wisconsin	35(14.81)	3(7.57)	42(inf)	40(23.6)	38(0.49)	40(1.5)	31(1.7)	N/A
Stanford	39(1.71)	0(0.34)	39(4.03)	39(inf)				

one is TMEM135-CCDC67: A total of 8 samples were found positive. In addition, high positive rate of CCNH-C5orf30 was also found in the prostate cohort from University of Wisconsin. In general, the rates of fusion gene positive samples are comparable among the 3 cohorts (table 1). Subsequent analyses showed that MAN2A1-FER (or normalized MANA1-FER), TRMT11-GRIK2, and mTOR-TP53BP1 gene fusions have the highest odd ratios for predicting the recurrence of prostate cancer for UPMC and University of Wisconsin cohorts (Table 2).

Table 3

Model	Fusion genes only					2x2 table		
	Sensitivity	Specificity	Youden	Accuracy	AUC		Recurrent (n=107)	Non-Recurrent (N=164)
RF	0.80	0.81	0.61	0.81	0.862	Positive	TP=86	FP=31
	Top 6, cutoff=0.2					Negative	FN=21	TN=133
SVM	0.71	0.87	0.58	0.81	0.77	Positive	TP=76	FP=21
	Top 4, cutoff=0.2					Negative	FN=31	TN=143
LDA	0.71	0.88	0.59	0.81	0.85	Positive	TP=76	FP=20
	Top 6, cutoff=0.4					Negative	FN=31	TN=144

To establish a prediction model, we combined top 6 fusion genes that have prediction power to construct classification models to predict prostate cancer recurrence. As shown in table 3, all three models (Random Forest, Support vector machine and Linear discriminant analysis) yielded very similar accuracy: 81%, even though the specificity and sensitivity may vary. When combined with gleason's score and TNM pathology staging, the accuracy improves to 84-86%.

When the same models were applied to the dataset from University of Wisconsin, the accuracy rate yielded 75-84%. Interestingly, when combined with Gleason's grade and pathology TNM staging, the accuracy rate improved to 88-90%. However, the same model fared worse in Stanford data set: 67-68% accuracy was found. Combination with fusion genes, Gleason's grade and pathology TNM staging improves the accuracy to 75%.

Table 4

Model	Gleason and TNM stage + fusion genes					2x2 table		
	Sensitivity	Specificity	Youden	Accuracy	AUC		Recurrent (n=208)	Non-Recurrent (N=368)
RF	0.79	0.82	0.61	0.81	0.86	Positive	TP=164	FP=67
	top 5 fusion genes, cutoff=0.2					Negative	FN=44	TN=301
SVM	0.75	0.83	0.59	0.81	0.84	Positive	TP=157	FP=61
	Top 6 fusion genes, cutoff=0.2					Negative	FN=51	TN=307
LDA	0.77	0.85	0.61	0.82	0.87	Positive	TP=160	FP=57
	Top 5 fusion genes, cutoff=0.4					Negative	FN=48	TN=311

When all data were pooled, and 10 fold cross validation was performed. The accuracy of fusion gene prediction rate is 76-77%. When combined fusion genes, Gleason's grading and TNM staging, the accuracy is improved to 81-82% (table 4). In contrast, if prediction is only relied on Gleason's grade and TNM staging, the prediction accuracy is 74-76% across all three data set. As a result, we concluded that fusion contains independent prediction value and can assist in predicting the clinical outcomes of prostate cancer.

Subtask 3: A training model using fusion genes have been established. It appears that the microdissecting samples from UPMC performed the best. Thus, there is some concern on the impact of purity of the cancer samples from Stanford and University of Wisconsin due to uncertainty nature of the needle core samples in terms of % cancer in the samples. The lesson from the project is that we should have unified the sample standard in the very beginning. To address these issues, this join plan is to unify the standard to microdissection so that the results are comparable among the three institutes, and improve the

prediction results. In addition, additional methods to examine the fusion genes are proposed to verify the Taqman and Sanger sequencing methods. Other types of samples will be submitted as controls of the assays. As detailed in previous progress reports, we have been investigating whether fusion transcripts described previously are prognostic in localized prostate cancer. The investigators have had ongoing discussions regarding concerns over possible artifacts in the RT-PCR-based assay of these fusions that had temporarily reached an impasse late last fall. The investigators have had considerable correspondence and communications to resolve this impasse and have jointly developed a plan to evaluate and validate the performance of the RT-PCR assay. The experiments agreed upon are outlined below. All the investigators have agreed to the following plan to be carried out at the University of Pittsburgh.

TAQMAN QRT-PCR analysis for the MAN2A1-FER, TRMT11-GRIK2, MTOR-TP53BP1, CCNH-C5orf30, KDM4-AC011523.2, SLC45A2-AMACR, TMEM135-CCDC67 and LRRC59- FLJ60017 fusion genes will be carried out at the CLIA certified lab at the University of Pittsburgh using approximately 200 samples provided by Drs. Brooks and Jarrard. These samples will include negative controls such as human foreskin and non-mammalian samples, normal prostate tissue samples, as well as recurrent and non-recurrent tumors (all blinded). All the normal and cancer tissue samples will be microdissected using H&E stained slides and a scalpel to ensure that they are as pure as possible. The timeline for tissue submission is 50 samples for 3 months from each institute. Thus, the tissue submission will be completed in the first 6 months of the funded period. All Taqman and Sanger sequencing results will be available once the analyses are completed the submission of the tissues. Preliminary analysis will be available shortly after the completion of experiments and the clinical information is obtained. Quarterly progress report will be submitted to DOD.

Once the experiments have been completed, Dr. Luo will provide primary data to Dr. Brooks and Jarrard who will then break the code. A subset of samples (approximately 50) that have been found to be positive for gene fusions, along with appropriate controls, will be further validated by FISH analysis. If necessary, the results can also be validated for a small number of samples by deep sequencing; since this method is very expensive, a source of funds will need to be identified for this purpose. Fifty percent of the results of FISH will be available 6 months after the resumption of the funding. All results will be available 9 months after the resumption of the funding. Deep sequencing will be performed once new funding is identified. The analyses results will be available 2 months after the sequencing.

Once the above experiments have been completed, all the investigators will meet in Pittsburgh to review and discuss the data and devise a plan for future studies. This meeting will be facilitated by the leadership of the University of Pittsburgh School of Medicine.

Progress: Investigators in University of Pittsburgh, Stanford University and University of Wisconsin Madison, had conducted thorough and constructive conversation on the fusion gene test. A consensus had been reached on the selection of samples. Instead of pursuing high number of samples for model validation, we will increase the breadth of the investigation by including other methodologies in the investigation, including FISH and additional RNA sequencing of prostate cancer samples. Dr. Jarrard had submitted 58 samples for the fusion gene analysis. The assays were completed and clinical information was provided. Analysis is ongoing in this sample set, but globally fusions appear more common in the tumor than associated normal tissue. Some fusions are seen commonly in the associated normal tissue (MAN2A1-FER 72%, SLC45A2-AMACR 45%). No fusions for TMEM135-CCDC67 and KDM4B-AC011523.2 were seen in this dataset.

Dr. Brooks had submitted 90 blinded cases for the analysis. We have just completed the first round of analysis. Once the verification round of analysis is completed, the results will be tabulated. We will submit the results to Dr. Brooks for de-identification and to obtain the clinical information of the samples. We have also analyzed the fusion transcripts on 90 samples from UPMC cohort for the validation purposes. The prediction analysis is on-going. All these analyses have been conducted in a blind fashion so that no information of the samples is disclosed to people who conduct the experiments. We have selected 20 samples from UPMC cohort to perform FISH analysis on MAN2A1-FER and SLC45A2-AMACR. The FISH probes have been constructed and labeled. Currently, we are carrying out FISH analysis in the InSitu lab of UPMC. We expect that we will have the results in several weeks.

Once all samples are de-identified, and the relevant clinical information is obtained, we will pool all the results of three cohorts to examine whether status or quantity of fusion genes plays a role in the aggressive behavior of prostate cancer. In addition, we may combine the status of fusion genes with other clinical parameters to evaluate whether fusion transcripts enhance the prediction of these clinical prediction models.

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?

Nothing to report

4. IMPACT:

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

MAN2A1-FER is the first tyrosine kinase fusion genes found to play critical roles in prostate cancer, it opens a new door for the treatment of prostate cancer using tyrosine kinase inhibitors. In addition, we developed a novel approach to treat human cancers that are positive for fusion gene by inserting a suicide gene into the chromosomal breakpoint of a fusion gene in the cancer genome. This could be a new way to treat prostate cancers that are refractory to other modes of the cancer treatment.

What was the impact on other disciplines?

Fusion transcripts mentioned in the proposed study was also present in ovarian cancer, breast cancer, colon cancer, lung cancer, liver cancer, GBM and esophageal adenocarcinoma, and may play roles in the developments of those cancers.

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:

Nothing to report

Changes in approach and reasons for change

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

Nothing to report

Changes that had a significant impact on expenditures

Nothing to report

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

Significant changes in use or care of human subjects

Nothing to report

Significant changes in use or care of vertebrate animals

Nothing to report

Significant changes in use of biohazards and/or select agents

Nothing to report

6. PRODUCTS:

Publications, conference papers, and presentations

Journal publications.

1. Yan-Ping Yu, Allan Tsung, Silvia Liu, Michael Nalesnik, David Geller, George Michalopoulos and **Jian-Hua Luo** (2019). Detection of fusion transcripts in the serum samples of patients with hepatocellular carcinoma. Oncotarget 10, 3352-3360.

2. Yan-Ping Yu, Peng Liu, Joel Nelson, Ronald L. Hamilton, Rohit Bhargava, George Michalopoulos, Qi Chen, Jun Zhang, Deqin Ma, Arjun Pennathur, Michael Nalesnik, George Tseng and **Jian-Hua Luo** (2019). Identification of recurrent fusion genes across multiple cancer types. Scientific Reports 9:1074. https://doi.org/10.1038/s41598-019-38550-6.

Books or other non-periodical, one-time publications.

Nothing to report

Other publications, conference papers and presentations.

J Luo, G Michalopoulos, Y Yu. Identification of recurrent fusion genes across multiple cancer types. The FASEB Journal 33 (1_supplement), 802.32-802.32.

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: *Zuoxiao Shi*
Project Role: *Research specialist*
Researcher Identifier (e.g. ORCID ID): *None*
Nearest person month worked: *9*
Contribution to Project: *Shi conduct H&E staining and clinical sample microdissection*

Name: *Nathan Damaschke*
Project Role: *PhD student*
Researcher Identifier (e.g. ORCID ID): *None*
Nearest person month worked: *4*
Contribution to Project: *Damaschke conduct clinical sample microdissection*

Name: *Tyler Etheridge*
Project Role: *Medical student*
Researcher Identifier (e.g. ORCID ID): *none*
Nearest person month worked: *4*
Contribution to Project: *Etheridge collect patients' information and requested tissue blocks*

Name: *Joseph Gawdzik*
Project Role: *Post-doctoral fellow*
Researcher Identifier (e.g. ORCID ID): *None*
Nearest person month worked: *18*
Contribution to Project: *Dr. Gawdzik conduct most clinical sample microdissection*

Name: *Bing Yang*
Project Role: *Researcher*
Researcher Identifier (e.g. ORCID ID): *None*
Nearest person month worked: *21*
Contribution to Project: *Dr. Yang conduct clinical sample microdissection, H&E staining, sample organization and coordinate with other institutions*

Name: *David F Jarrard*
Project Role: *Professor*
Researcher Identifier (e.g. ORCID ID): *None*
Nearest person month worked: *7*
Contribution to Project: *Dr. Jarrard oversaw the project.*

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

Nothing to report

What other organizations were involved as partners?

Nothing to report

8. **SPECIAL REPORTING REQUIREMENTS**
   COLLABORATIVE AWARDS:
   QUAD CHARTS:
9. **APPENDICES:**