Clinical Validation of a Serum Protein Panel (FLNA, FLNB and KRT19) for Diagnosis of Prostate Cancer

Shobha Ravipaty1, Wenfang Wu1, Aditee Dalvi1, Nikunj Tanna1, Joe Andreazi1, Tracey Friss1, Allison Klotz1, Chenchen Liao1, Jennifer Garren1, Sally Schofield1, Eleftherios P Diamandis2, Eric A Klein3, Albert Dobi4, Shiv Srivastava4, Poornima Tekumalla1, Michael A Kiebish1, Vivek K. Vishnudas1, Rangaprasad Sarangarajan1, Niven R Narain1, Shiv Srivastava4 and Viatcheslav R Akmaev*1

1Berg, LLC, 500 Old Connecticut Path Framingham, MA 01701, USA
2Department of Pathology and Laboratory Medicine, Mount Sinai Hospital, Toronto, Ontario, Canada
3Department of Surgery, Center for Prostate Disease Research, Uniformed Services University of the Health Sciences and the Walter Reed Military Medical Center, Bethesda, Maryland, USA
4Berg, LLC, 500 Old Connecticut Path Framingham, MA 01701, USA

Abstract

This study reports on the development of a novel serum protein panel of three prostate cancer biomarkers, Filamin A, Filamin B and Keratin-19 (FLNA, FLNB and KRT19) using multivariate models for disease screening and prognosis. ELISA and IPMRM (LC-MS/MS) based assays were developed and analytically validated by quantitative measurements of the biomarkers in serum. Retrospectively collected and clinically annotated serum samples with PSA values and Gleason scores were analyzed from subjects who underwent prostate biopsy, and showed no evidence of cancer with or without indication of prostatic hyperplasia, or had a definitive pathology diagnosis of prostatic adenocarcinoma. Probit linear regression models were used to combine the analytes into score functions to address the following clinical questions: does the biomarker test augment PSA for population screening? Can aggressive disease be differentiated from lower risk disease, and can the panel discriminate between prostate cancer and benign prostatic hyperplasia? Modelling of the data showed that the new prostate biomarkers and PSA in combination were better than PSA alone in identifying prostate cancer, improved the prediction of high and low risk disease, and improved prediction of cancer versus benign prostatic hyperplasia.

Keywords: Prostate cancer; Prostate specific antigen; Biomarker; FLNA; FLNB; KRT19

Abbreviations: PSA: Prostate Specific Antigen; GS: Gleason Score; FLNA: Filamin-A; FLNB: Filamin-B; KRT19: Keratin-19; IPMRM: Immunoprecipitation Multiple Reaction Monitoring)

Introduction

Prostate cancer (PrCa) is a leading cause of cancer deaths in American men [1,2]. Currently the most prevalent method for detection of PrCa includes screening with a prostate-specific antigen (PSA) blood test followed by a digital rectal examination and diagnostic prostate biopsy. The PSA-based test is first-line for screening of PrCa [3,4]. However, in 2012 the USPTF issued a recommendation against use of PSA-based screening due to its limitations in accuracy. Use of this test has resulted in over-diagnosis and overtreatment of nonlethal cancers, resulting in reduced quality of life of patients who did not require treatment [4]. Thus, there is a need for a diagnostic test with increased predictive power which may reduce the frequency of biopsy, over detection and overtreatment of the 40% to 50% of current cases that are indolent [3].

Recent advances in PrCa detection include: tests that measure variants of PSA in blood [5], expression or hypermethylation of PrCa specific genes [6], or proteins in biopsy tissues [7], and expression of PrCa associated genes [8], or fusion genes in post-digital rectal examination urine [9], or urine exosomes [10]. Markers such as the TMPRSS2-ERG fusion and PCA3 have improved diagnostic accuracy, although discrimination between low risk and more aggressive disease states remains challenging. Indeed, there is a continued need for core biopsy specimens to confirm diagnosis [3].

Therefore, improvements in identification of high-risk cancers from low risk cancers need to be developed when diagnosing prostate cancer, in order to administer the appropriate course of action [10].

High-risk prostate cancers (with a Gleason score of at least 7) have been shown to be more accurately identified using the STHLM3 model, which suggests that structured screening that includes clinical factors, PSA and some PSA derivatives, and germline allelic variants, could reduce the number of prostate biopsies by about a third, when compared to PSA alone [11]. Similarly, a study in 2012 showed that a blood-based Biomarker Panel (CRTAM, CXCR3, FCRL3, KIAA1143, KLF12, TMEM204) could identify men with aggressive prostate cancer, thereby reducing the over-diagnosis and overtreatment that currently results from using PSA alone [12]. These and other studies demonstrate the improvements that have been made in predicting aggressive cancers. Nevertheless, there remains a need for better stratification of patients to low and high-risk forms of the disease.

This study utilizes a novel panel of serum biomarkers to augment the diagnosis of prostate cancer in conjunction with the PSA test. The biomarkers described herein are novel entities and are not PSA derivatives. The panel was developed by using a multomic approach that defined filamin-A (FLNA), filamin-B (FLNB), and keratin-19 (KRT19) in the panel [13]. New ELISAs for FLNA and FLNB were developed along with immunoprecipitation multiple reaction

*Corresponding author: Viatcheslav R Akmaev, Berg, LLC, 500 Old Connecticut Path, Framingham, MA 01701, USA, Tel: 6175882235; E-mail: slava.akmaev@berghealth.com

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monitoring (IPMRM) for FLNA, which resulted in significant improvements in the context of PrCa detection and prognosis.

Materials and Methods

Human and animal rights

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

Quantitation of FLNA in human serum samples by ELISA

Antibodies against FLNA were developed by immunizing mice with E. coli-expressed partial FLNA protein (aa 1443-2131). Hybridomas were selected by affinity to HEK293-expressed full length FLNA by ELISA and BLI (Bio-layer interferometry). Antibodies were further screened to ascertain no reactivity to Filamin family members FLNB and FLNC. Sandwich ELISA was optimized by R&D Systems using two high affinity FLNA antibodies (3F4 and 6E3 developed by Berg, LLC in-house) and HEK293-expressed full length FLNA as a calibrator.

Quantitation of FLNB in human serum samples by ELISA

Antibodies against FLNB were developed by immunizing mice with E. coli-expressed partial FLNB protein (aa 1416-2089). Hybridomas were selected by affinity to HEK293-expressed full length FLNB by ELISA and BLI (Bio-layer interferometry). Antibodies were further screened to ensure no cross-reactivity to the other two filamin family members, FLNA and FLNC. Sandwich ELISA was optimized by R&D Systems using two high affinity FLNB antibodies (3F10 and 5H7 produced in-house) and HEK293-expressed full length FLNB as calibrator.

FLNA and FLNB assay validation

The following parameters were assessed during assay validation: Calibration curve precision and accuracy were evaluated using 4-PL non-linear regression model over 6 assay runs. Intra and inter-run QC precision was evaluated over 6 separate daily runs using both lyophilized QC’s and serum QC’s (n ≥ 12). Short-term stability was evaluated for up to 24 hours at 4°C, benchtop and 37°C. Freeze-thaw stability was assessed for up to five cycles at -80°C. Spike recovery was evaluated at mid (25 ng/ml) and high (50 ng/ml) analytical range of the assay.

Quantitation of KRT19 in human serum samples by ELISA

KRT19 was assessed using a commercially available diagnostic ELISA kit per manufacturer’s instructions (Fujirebio, Malvern, PA). This kit detects the CYFRA-21-1 fragment of KRT19. Manufacturer’s instructions were followed for sample testing.

KRT19 assay validation

The following parameters were assessed during assay validation: Calibration curve precision and accuracy were evaluated using suggested 4-degree polynomial regression model over 5 assay runs. Intra and inter-run QC precision was verified over 6 separate daily runs using both lyophilized QC’s and serum QC’s (n ≥ 12). Short-term stability was evaluated for up to 24 hours at 4°C, benchtop and 37°C. Freeze-thaw stability was assessed for up to five cycles at -80°C. Spike recovery was evaluated at mid (25 ng/ml) and high (50 ng/ml) analytical range of the assay.
water (ThermoFisher Scientific) and mobile phase B consisted of 0.1% formic acid in acetonitrile (ThermoFisher Scientific). The gradient with respect to %B was as follows: 0 to 1.5 min, 5%; 1.5 to 2 min, 5% to 15%; 2 to 5 min, 15%; 5 to 7.1 min, 15% to 20%; 7.1 to 8.1 min, 20% to 80%; 8.1 to 9.0 min, 80%; and 9.0 to 9.1 min, 80% to 5%. 9.1 to 16 min, 5%. The instrument parameters for 6500 QTRAP mass spectrometer were as follows: Ion spray voltage of 5500 V, curtain gas of 20 psi, collision gas set to “medium”, interface heater temperature of 400°C, nebulizer gas (GS1) of 80 psi and ion source gas (GS2) of 80 psi and unit resolution for both Q1 and Q3 quadrupoles.

Selection of surrogate peptides and MRM transitions: Potential surrogate peptides for FLNA quantitation were initially chosen by Skyline software [14] and LC-MS/MS analysis (LTQ Orbitrap Velos coupled to Eksigent nano-LC) of recombinant FLNA protein (GenScript) tryptic digest. From the list of potential surrogate peptides, two surrogate peptides, peptide 2 (AGVAPLQVK) and peptide 4 (YNEQHVPGSPFTAR) were selected based on surrogate peptide selection rules [15] and signal intensities of the peptides in spiked and unspiked serum digests. The uniqueness of the surrogate peptides to the target protein was confirmed by running BLAST searches. Heavy labeled versions of the surrogate peptide 2 and 4, AGVAPLQVK and peptide 4 (YNEQHVPGSPFTAR) were then synthesized and used as internal standards. MRM transitions were optimized using synthetic surrogate peptides (GenScript) and their internal standards (ThermoFisher Scientific) and the following m/z transitions were monitored: P2, 441.7 (M+2H)2+→584.5 (y5+); P4 535 (M+3H)3+→842.4 (y8+); P2_IS 445.5 (M+2H)2+→592.1 (y5+); P4_IS, 538.4 (M+3H)3+→842.5 (y8+).

IPMRM data analysis and quantitation
Data analysis was performed using the Analyst® software (version 1.6.2, AB Sciex, Framingham, MA) and peak integrations were reviewed manually. The calibration curve for FLNA P2 and P4 peptides was constructed by plotting the peak area ratios (analyte/ internal standard) versus concentration of the standard with 1/x2 linear least square regression. The regression equations from P2 and P4 calibration standards was used to back-calculate the measured P2 and P4 concentrations for each QC and unknown sample.

Assay validation
The following parameters were assessed during assay validation: Calibration curve linearity and linearity regression weighting factor were assessed from 4 independent calibration curves for P2 and P4. Intra and inter-batch precision of the assay was evaluated by analyzing low QC (LQC) and high QC (HQC) human serum samples (6 replicates each) on different days. LQC and HQC samples were also used to assess sample stability in the autosampler (4°C stored up to 48 hours), short term stability (at 4°C and ambient temperature, stored up to 48 hours), long term stability (at -80°C), and freeze-thaw stability (up to three cycles at -80°C and -20°C). Potential interferences were evaluated by spiking HQC and LQC samples with hemoglobin (500 mg/dL), unconjugated bilirubin (30 mg/dL), and triglycerides (1000 mg/dL). In addition, carry over and instrument drift were also assessed.

Sample collection
Clinically annotated serum samples with PSA values and Gleason scores (GS) were collected from males visiting Mount Sinai Hospital, Toronto, Canada between September 2007 and April 2008 with prostatic symptoms. Samples were collected just before biopsy from 662 patients who underwent prostatic biopsy that resulted in definitive diagnosis of prostatic conditions including prostate cancer (n=311), benign conditions (n=122), atypical small acinar proliferation (n=26), inflammation (n=58), prostatic intraepithelial neoplasia (n=69), microfocus adenocarcinoma (n=16), and benign prostatic hyperplasia (n=60). A total of 503 samples were included in the final analysis; 159 samples were removed from analysis due to missing variable values in either FLNA, FLNB, P2, or P4.

Statistical analysis
Regression models were built and compared for their ability to classify patients with prostate cancer with low GS (≤6), high GS (≥7), and with an absence of cancer on biopsy. The resulting Prostate Cancer Panel predictive algorithms were based on the regression models and probability threshold values selected to achieve a certain level of test sensitivity or specificity. All analyses were performed in R 3.2.2 with significance level of 0.05, unless otherwise stated.

Results
This study describes the development of novel biomarker assays to screen for and monitor prostate cancer in concert with the existing PSA test. Two ELISAs were developed for the new biomarkers FLNA and FLNB. The existing commercial assays were found to perform suboptimally. The other biomarker in the panel, KRT19, already had a commercially available ELISA kit for use. Our preliminary investigations on the quantitation of FLNA, FLNB and KRT19 using an MRM-only approach proved to be futile due to insufficient sensitivity. Since our novel biomarkers were low abundance proteins in human plasma/serum matrices, development of quantitative mass spectrometry based assays was particularly challenging. To improve the sensitivity of the assay, an immunoaffinity enrichment approach coupled with MRM was evaluated using the antibodies developed for ELISA. Of the three biomarkers, an IPMRM assay was feasible only for FLNA biomarker in serum matrix.

FLNA, FLNB and KRT19 ELISA validation
FLNA and FLNB ELISA method development involved optimization of several parameters including antibody pair selection, blocking buffers, assay diluents, incubation times, matrix selection, selectivity and sensitivity. The assay performance was then evaluated by performing a validation study in the serum matrix. Since the KRT19 assay was a commercial kit, validation of the assay was performed in-house. All ELISA assays met validation acceptance criteria as detailed in Table 1.

FLNA IPMRM assay development and validation
FLNA IPMRM assay development involved optimization of several parameters including: selection of antibodies, immobilization, immunoaffinity capture, incubation, elution, trypsin digestion and other mass spectrometry parameters. The performance of the FLNA IP-MRM assay was assessed using serum QCs and the assay met validation acceptance criteria as shown in Supplemental Table 1.

Comparison of the Prostate Cancer Biomarker Panel FLNA, FLNB and KRT19, versus PSA alone for prediction of prostate cancer
Sera from patients were then tested with the biomarker ELISAs and IPMRM. The results were combined with data on age, PSA, and Gleason score and subjected to regression modelling. Table 2 shows the patient demographic data pertaining to age, cancer status, GS and benign classification for the samples analyzed in this study. The Prostate Cancer Biomarker Panel, (biomarkers FLNA, FLNB, age
and PSA) improved the classification of prediction of prostate cancer over PSA alone (AUC = 0.64, [0.59, 0.69], vs 0.58) (Figure 1A). The predictive algorithm was set to have a cutoff = 0.45, which is based on the regression model achieving sensitivity equivalent to PSA = 4 ng/ml. The distribution of predicted probabilities for patients with and without PrCa are shown in Figure 2A.

Table 1: FLNA, FLNB and KRT19 ELISA validation summary. All levels reported in this table are within the appropriate acceptance criteria.
High-risk disease prediction with FLNB, age and PSA, compared with PSA alone

Comparing serum samples from patients with high GS (≥7) and samples from patients with low GS (≤6) with our biomarker FLNB, age and PSA, against the use of PSA alone is shown in Figure 1B. The model that achieved the greatest prediction between patients with GS ≤6 and patients with GS ≥7 is a regression model with the biomarker FLNB, age and PSA. The algorithm was set to have a cutoff=0.02, which is based on the regression model achieving sensitivity ≥0.95. When compared with PSA alone, regression modelling with FLNB, age and PSA improved the classification of low and high Gleason scores (AUC=0.81 [0.70, 0.90], vs 0.71). The distribution of predicted probabilities for patients with Gleason score ≤6 and Gleason score ≥7 are shown in a box plot (Figure 2B).

Low-risk disease prediction with FLNA, age and PSA, compared with PSA alone

Patient serum samples with Gleason scores ≤6 were analyzed with our biomarker FLNA, age and PSA, compared with PSA alone as shown in Figure 1C. The model that achieved the highest prediction for patients with low Gleason score (≤ 6) over PSA alone is a regression model with biomarker FLNB, age and PSA vs PSA alone. The predictive algorithm was set to have a cutoff=0.15, which is based on the regression model achieving sensitivity ≥0.8. The Biomarker Panel FLNB, age and PSA has improved classification over PSA alone (AUC 0.72 [0.66, 0.78] vs 0.63). The distribution of predicted probabilities for patients with low Gleason score are shown in a boxplot in Figure 2C.

Prediction of benign prostatic hyperplasia versus prostate cancer with FLNA, KRT19 and age, compared with PSA alone

Samples of patient sera were analyzed with the biomarkers FLNA, KRT19 and age combined, versus PSA alone. Figure 1D showed the highest prediction between patients with benign prostatic hyperplasia versus PrCa. The predictive algorithm was set to have a cutoff=0.86, which is based on the regression model achieving sensitivity ≥0.8. The biomarkers FLNA, KRT19 and age have improved classification over PSA alone (AUC=0.70 [0.60, 0.80], vs 0.58). The distribution of predicted probabilities for patients with benign prostatic hyperplasia versus PrCa is shown in Figure 2D.

Supplementary Table 2 summarizes the cutoff, AUC, sensitivity, specificity, positive predictive values (ppv), and negative predictive values (npv) of the predictive algorithms for each comparison.

Discussion

This manuscript describes the validation of a novel Biomarker Panel for prostate cancer screening using traditional ELISA and IPMRM for analysis of patient serum samples. IPMRM combines IP with mass spectrometry and allows the rapid quantitation of proteins with
enhanced sensitivity and specificity. For biomarkers, this technique has shown to achieve low ng/mL quantitation by selective enrichment of target proteins in complex matrices [16-18].

Currently, there is an unmet clinical need for a more specific and accurate test for prostate cancer. The standard of care for prostate cancer diagnosis is the PSA test in combination with diagnostic prostate biopsy. However, the PSA test has a high false positive rate and may not reflect true cancer detection. In 2012, the US Preventive Services Task Force issued a recommendation against the use of PSA screening due to the over-detection and overtreatment of non-lethal cancers [3]. To confirm diagnosis, patients undergo invasive prostate biopsies in this population.

In this study, over 500 serum samples were screened against the PrCa Biomarker Panel (FLNA, FLNB, KRT19), and this data was combined with age, PSA test results and Gleason score to assess whether this combinatorial approach was better at predicting prostate cancer, high-risk disease versus indolent disease and discriminating between benign prostate hyperplasia and cancer than the PSA test alone. Data was analyzed by regression modelling. The PrCa biomarkers FLNA, FLNB, age and PSA predicted the likelihood of a patient having prostate cancer better than PSA alone. This was an improvement over the standard PSA test, and could reduce the number of unnecessary biopsies in this population.

The primary goal of this study was to develop sensitive, specific and reliable assays to quantitate the biomarkers FLNA, FLNB and Keratin-19 in serum and evaluate their clinical utility. The identification of biomarker panels for health problems such as cancer are being used more frequently to address the need to better classify disease groups, predict the effect of therapeutic intervention, and monitor and detect cancer as early as possible [21]. Biomarkers are most often identified as multiple protein panels [22], which then must be verified and validated. Biomarkers can be identified in the low ng/ml range in an MRM multiplex assay, which minimizes assay time and sample volume required [23].

This study addresses the development and clinical validation of a novel biomarker panel for improving the detection of prostate cancer. A recent study by this group discovered three novel biomarkers, FLNA, FLNB and KRT19 for prostate cancer [13,24]. The Prostate Cancer Biomarker proteins in the panel have been shown in previous studies to have links to cancer. Previous work has shown the absence of KRT19 in prostate cancer cells compared to the levels observed in androgen refractory cell lines. This is suggestive of the utility of KRT19 as a biomarker for differentiating aggressive, metastatic forms of PrCa. Additionally, altered levels of KRT19 expression have been demonstrated in the bone marrow of metastatic PrCa patients [25]. FLNA and FLNB belong to a family of large actin-binding filamins and play a major role in cell migration, vascular development, extracellular signaling, and activity of integrins [26]. FLNA has been described previously as being involved in normal prostate physiology and in PrCa metastases [26-28]. FLNB was also shown to be involved in tumor growth and metastases [29].

New assays were developed for FLNA (ELISA and IPMRM) and FLNB (ELISA). The IPMRM assay was especially suited for detection of FLNA in serum, which is more abundant and has been detected in cleaved fragments [30]. ELISA alone may not detect all forms of FLNA in a serum sample. IPMRM allows detection of different peptides along the length of the entire protein. IPMRM assays were not developed for FLNB and KRT19 as both proteins were low in abundance in the serum samples.

In this study, over 500 serum samples were screened against the PrCa Biomarker Panel (FLNA, FLNB, KRT19), and this data was combined with age, PSA test results and Gleason score to assess whether this combinatorial approach was better at predicting prostate cancer, high-risk disease versus indolent disease and discriminating between benign prostate hyperplasia and cancer than the PSA test alone. Data was analyzed by regression modelling. The PrCa biomarkers FLNA, FLNB, age and PSA predicted the likelihood of a patient having prostate cancer better than PSA alone. This was an improvement over the standard PSA test, and could reduce the number of unnecessary biopsies in this population.

The biomarker panel FLNB, age and PSA showed improved sensitivity and specificity over the use of PSA alone in predicting whether patients had Gleason score ≥7 or lower Gleason score ≤6. Additionally, the biomarkers FLNA, KRT19 and age were able to improve the classification of whether patients had benign prostatic hyperplasia or cancer over the PSA test alone. Current commercially available tests include the 4K score [31], which has been extensively tested in Europe and the US, and discriminates between high-risk and low-risk disease. This test utilizes the four-kallikrein (KLK) panel immunoassay of KLK2, total PSA, intact PSA, and free PSA in combination with a patient’s age, DRE results and prior biopsy status. This information is analyzed by an algorithm to determine the percentage risk for aggressive prostate cancer. 4Kscore test was used in a prospective validation study in the US in 2014, and performed well in identifying patients with high risk disease. However, this test still requires a prostate biopsy and is heavily dependent on PSA levels. In
comparison, the test described in this manuscript with FLNA, FLNB and KRT19, does not require invasive procedures to be performed on patients, and is also able to distinguish between benign prostatic hyperplasia and prostate cancer. The PHI (prostate health index) test also discriminates between high and low risk cancers. PHI was shown in a European study to be more accurate than PSA alone in predicting prostate cancer in obese patients [32]. However, it is unclear at present if PHI can discriminate between intermediate PSA values [33-35].

Prostate cancer antigen 3 (PCA3) is a non-PSA-based test of the expression of long non-coding RNA that is elevated in over 90% of PrCa tissue, but is not found in BPH or healthy tissues [36]. This is a non-invasive urine test that in combination with PSA improves PrCa prediction [37]. The androgen-induced transmembrane protease, serine 2 (TMPRSS2-ERG) is detected in urine samples of suspected PrCa patients. However, TMPRSS2-ERG is absent in 50% of cancers, and therefore it must be multiplexed with other biomarkers such as PCA3 [38]. In a study of 1300 men combination testing with TMPRSS2-ERG and PCA3 improved the sensitivity of PrCa diagnosis [39]. However, both PCA3 and TMPRSS2-ERG are dependent on relative PSA expression diagnosis [39,38]. The biomarker alpha-methylacyl-CoA racemase (AMACR), detected by RNA expression profiling demonstrates high sensitivity and specificity in prostate biopsy tissue [40]. However this biomarker is not specific to prostate cancer, nor can it be used for detection of invasive cancer in urine [41], but can be used when prostate biopsy analysis is ambiguous [42]. In conclusion, the Prostate Cancer Biomarker Panel developed in this study demonstrates an advantage over existing tests in that it not only discriminates between high and low-risk disease, it also discriminates between cancer and benign prostatic hyperplasia. Use of these biomarkers will potentially allow for more accurate diagnostic and treatment decisions, and improve the accuracy of disease prognosis by better distinguishing between indolent and high-risk disease.

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Conflict of Interest

SR, WW, AD, NT, JA, TF, AK, CL, JG, SS, PT, MK, VV, RS, NN and VA are current or previous employees of BERG, LLC and have stock. NN is also co-founder for writing and editing assistance, and Jiaqi Qu for help with data analysis. This manuscript are those of the authors and do not reflect the official policy of the Army, Department of Defense or the U.S. government. This manuscript are those of the authors and do not reflect the official policy of the Army, Department of Defense or the U.S. government. This manuscript are those of the authors and do not reflect the official policy of the Army, Department of Defense or the U.S. government.

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