Phosphorylation of Akt (Ser\textsuperscript{473}) is an Excellent Predictor of Poor Clinical Outcome in Prostate Cancer

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ABSTRACT

We previously showed, by immunohistochemistry with phospho-specific antibodies, increased phosphorylation (activation) of Akt (Ser\textsuperscript{473}) [phosphorylated Akt (pAkt)] in high-Gleason grade prostate cancer (Malik SN, et al., Clin Cancer Res 2002;8:1168–71). Elevation of pAkt was accompanied by decreased phosphorylation of extracellular signal-regulated kinase (ERK) (1/2 (Thr\textsuperscript{202}/Tyr\textsuperscript{204}) [phosphorylated ERK (pERK)], indicative of inactivation. In this report, we determined whether increased pAkt and decreased pERK predicted clinical outcome. Prostate-specific antigen (PSA) failure (detectable and rising PSA) versus PSA non-failure (undetectable PSA 5 years after prostatectomy) was used as a surrogate for clinical outcome. Prostate tumors from cases of PSA failure versus non-failure were stained for pAkt and pERK. A significant increase in mean pAkt staining (P < 0.001) in the PSA failures versus non-failures was seen based on the Wilcoxon signed ranks test (222.18 ± 33.9 \(n=37\) versus 108.79 ± 104.57 \(n=16\)). Using the best-fitting multiple logistic regression equation, a 100-point increase in pAkt staining resulted in a 160\% increase in the odds of being a PSA failure. There was decreased staining for pERK in PSA failures versus non-failures: a 100-point decrease resulted in an 80\% increase in the odds of being a PSA failure. Each of these effects assumed the other biomarker was held constant. The area under the receiver-operating characteristic curve for these two biomarkers predicting PSA failure was 0.94, indicating excellent discrimination between PSA failure and non-failure cases. These data indicate that increased pAkt, alone or together with decreased pERK, is an important predictor of probability of PSA failure. However, pERK alone was not a significant predictor of PSA failure.

INTRODUCTION

Prostate cancer is the most commonly diagnosed cancer in men, accounting for 30\% of all cancers, and is second only to lung cancer as a leading cause of cancer deaths in men (1, 2). In 2003, an estimated 220,900 American men were diagnosed with prostate cancer, and approximately 28,900 men died from the disease (3). These figures suggest that not all prostate cancers are aggressive; however, current methods of diagnosis including screening for high serum levels of prostate-specific antigen (PSA) and pathological grading of prostate biopsies cannot precisely distinguish between clinically aggressive and clinically indolent forms of prostate cancer. Therefore, approximately four times as many men are treated for the disease than would have died from it if left untreated (4). Thus, there is an urgent need to identify biomarkers that distinguish the clinically aggressive forms of the tumor from the clinically indolent ones.

Virtually all prostate cancers are initially androgen dependent. Most tumors are primarily treated with surgery or radiation therapy. Advanced-stage disease at initial diagnosis or disease progression after primary treatment is treated with androgen ablation therapy (5–7). Unfortunately, with the passage of time, virtually all tumors treated with antiandrogen therapy will become androgen resistant (2). Despite being androgen resistant, the tumor continues to express the androgen receptor (8, 9) and androgen-regulated genes such as PSA, which indicates that the androgen signaling pathway remains intact (8). Androgen-independent prostate cancer is likely to progress and metastasize and therefore has a low survival rate (3). There is currently no consensus on therapy for androgen-independent prostate cancer.

We have shown previously, by immunohistochemistry, increased phosphorylation (activation) of the serine/threonine kinase Akt (Ser\textsuperscript{473}) [phosphorylated Akt (pAkt)] in poorly differentiated prostate cancer (10). In contrast, there was decreased phosphorylation of extracellular signal-regulated kinase (ERK; Thr\textsuperscript{202}/Tyr\textsuperscript{204}) [phosphorylated ERK (pERK)], a downstream effector of the Ras/mitogen-activated protein kinase pathway, in poorly differentiated prostate cancer, indicative of inactivation (10). In confirmation of these observations, Paweletz et al. (11) reported by protein microarray analysis that prostate cancer progression was associated with increased protein kinase B/Akt phosphorylation and decreased phosphorylation of ERK.

In this study, we investigate whether increased pAkt and/or decreased pERK could be a predictor of poor clinical outcome. With PSA failure and PSA non-failure as a surrogate for clinical outcome, we show that elevated pAkt, alone or together with decreased pERK, is an excellent predictor of PSA failure. Other commonly used indicators for predicting disease recurrence, including the cell proliferation antigen Ki67 and Gleason grading, are also compared as biological markers for clinical outcome.

MATERIALS AND METHODS

Patients. Prostate cancers were obtained after radical prostatectomy from men (age range, 42–81 years; mean age, 64.5 years) including 33 white, 15 Hispanic, and 5 African Americans, reflecting the ethnic distribution of patients seen at the South Texas Veterans Health Care System, Audie Murphy Veterans Administration Hospital, and the University Hospital, San Antonio, Texas. Clinical data were obtained from the Tumor Bank for Prostate Cancer, Department of Pathology, University of Texas Health Science Center at San Antonio under an Institutional Review Board-approved protocol. PSA data available from patient follow-up were evaluated to assess “good” and “poor” outcome after radical prostatectomy. Cases with a poor outcome were defined as follows: PSA was detectable and rose to >0.5 ng/ml within 60 months after radical prostatectomy; and a second value obtained confirmed the elevated PSA. The patient was also included if he had no second value but was judged by his physician to have recurrent disease and was treated for recurrent disease without the second PSA. Cases with a good clinical outcome were defined as follows: (a) the PSA remained <0.3 ng/ml for at least 60 months; and (b) there was no other evidence of recurrent disease at time of selection. Sections were chosen for analysis when at least 30\% of the slice surface area was involved by tumor. Slides for immunohistochemical analysis were not selected based on Gleason grade or any other known factor. Tissue arrays were prepared using a 4-mm punch biopsy, placing approximately 20 cores in each block. Gleason grade scoring was performed in tumors at the time of prostatectomy (12).

Antibodies. Mouse monoclonal Ki67 antigen clone MIB-1 was obtained from DAKO (Carpinteria, CA); polyclonal anti-pAkt (Ser\textsuperscript{473}) antibody and polyclonal anti-pERK (Thr\textsuperscript{202}/Tyr\textsuperscript{204}) antibody were obtained from Cell Sig-
naling Technology (Beverly, MA); and polyclonal anti-Akt1/2 antibody (H-136) and polyclonal ERK1 antibody (C-16; also stains ERK2) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

Immunohistochemistry. Immunohistochemical studies were conducted as described previously (10). Briefly, sections were heated to 60°C and dehydrated in xylene and graded alcohols. Antigen retrieval was performed with 0.01 M citrate buffer at pH 6.0 at 95°C. Sections were incubated in primary antibody diluted in 50 mM Tris-HCl (pH 7.6), 150 mM NaCl, and 0.1% Tween 20 containing 1% ovalbumin and 1 mg/ml sodium azide for 12 h, followed by incubations with biotinylated secondary antibody for 15 min, peroxidase-labeled streptavidin for 15 min (LSAB-2 System; DAKO), and diaminobenzidine and hydrogen peroxide chromogen substrate (DAKO) plus diaminobenzidine enhancer (Signet, Dedham, MA) for 10 min. Slides were counterstained with hematoxylin and mounted. The negative controls were incubated with immunoglobulin fraction (normal rabbit for pAkt, Akt, ERK, and pERK) in place of polyclonal primary antibody. The positive control for Ki67 is normal mouse immunoglobulin. The positive control for pAkt, Akt, and ERK was LNCaP cells. PC-3 cells were used as positive control for pERK, and human appendix served as a positive control for Ki67.

Semiquantitative Analysis. The degree of staining was evaluated blindly by a pathologist. For pERK, ERK, Akt, and pAkt, total staining was scored as the product of the staining intensity (on a scale of 0–3) × the percentage of cells stained, resulting in a scale of 0–300. Staining intensity was scored as follows: 0, none of the cells stained positively; 1, weak staining; 2, moderate staining intensity; and 3, strong staining intensity (13). The Ki67 labeling index (LI) was determined by counting 500 cells and determining the percentage of cells that stained positively for Ki67. Positive controls were used with each staining run to identify problems with immunohistochemistry. Positive controls were also graded subjectively using the intensity scoring referred to above, and the staining was considered acceptable only when the positive control displayed staining intensity > 2 (on a scale of 0–3 reactivity). All assays demonstrating inferior reactivity on positive controls were examined and repeated. Importantly, all assays were stained at the same time with the same reagents.

Data Interpretation and Analysis. To compare the expression levels of pAkt, Akt, pERK, ERK, and Ki67 LI between the PSA failure cases and the non-failure cases, the t test and the nonparametric Wilcoxon test (equivalent to the Mann-Whitney test) were considered (14). Pearson’s and Spearman’s correlation coefficients were used to determine whether biomarkers were related to each other over all cases and within each of the PSA failure and non-failure cases. When assumptions for the normal distribution were not met, standard transformations, such as logarithm, were considered, as well as the nonparametric tests. Results are reported with methods whose assumptions are most appropriately met by the data. A uniformly accurate measure of the time to PSA failure was not available to provide survival analysis of these markers. Therefore, multiple logistic regressions were used to evaluate the joint ability of markers to predict which cases would become PSA failures and which cases would not, based on biomarkers considered here. The Hosmer-Lemeshow goodness of fit for χ² test was used to evaluate how well logistic regression models match the data (14). Two-way interactions of significant markers were considered as well.

RESULTS

pAkt and pERK in PSA Failure and Non-Failure Cases. We have shown previously that pAkt increases with increasing Gleason scores (10). We investigated whether pAkt increase correlated with clinical outcome. Tissue arrays were prepared from 60 cases of PSA failure and non-failure, and tumor was present for investigation in 53 of these. To determine Akt activation levels, the arrays were stained with rabbit polyclonal antibodies to pAkt (Ser⁴⁷³) and scored as described in “Materials and Methods.” Briefly, staining intensities (scale, 0–3) and percentage of tumor staining were determined independently, and total staining was expressed as a product of the two numbers (resulting in staining scale ≥ 0–300). Examples of strong (300) and weak (100) staining scores for pAkt are shown in Fig. 1, A and C. The data were then classified as null (0), weak (1–100), moderate (101–200), or strong (201–300) pAkt staining (Table 1). All of the cases exhibiting null pAkt staining were PSA non-failures, whereas the majority (91%) of those exhibiting strong pAkt staining were PSA failures. Statistical analyses revealed significant differences in mean pAkt staining between the PSA failures (222.18 ± 33.9; n = 37) and non-failures (108.79 ± 104.57; n = 16) based on the Wilcoxon signed ranks test (P < 0.001). Because the distribution was non-normal, this test was deemed appropriate for comparing these groups. Total Akt showed no significant difference between PSA failures and non-failures (222.37 ± 70.42 versus 194.44 ± 105.56, respectively; P > 0.3).

In contrast to pAkt, the majority of all cases showed weak staining for pERK (Table 1). Most of the cases exhibiting no (0) or weak (1–100) pERK staining were PSA failures (78.1% and 63.6%, respectively). Fig. 1, B and D, shows examples of weak (100) and strong (300) pERK staining. The mean staining intensity for pERK was 36.8 ± 75.5 for PSA failure and 59.1 ± 82.08 for PSA non-failure (P = 0.34). There was no difference in staining intensity for total ERK between PSA failures (281.08 ± 46.18) and non-failures (288.24 ± 33.21; P > 0.5).

Elevated pAkt in Combination with Decreased pERK Is a Better Predictor of PSA Failure. Next we determined statistically whether pAkt and pERK, alone or in combination, predicted PSA failure. Our logistic regression prediction plot shows what combinations of pERK and pAkt are particularly prognostic of PSA failure and non-failure. For each value of pERK (0–300), the predicted probability of PSA failure was calculated by logistic regression analysis and plotted against pAkt staining intensities (Fig. 2A). This figure indicates that for a given value of pERK staining, the predicted probability of PSA failure increases with pAkt staining. Based on the best-fitting logistic regression equation, a 100-point increase in pAkt (one-third of its range) would result in a 160% increase in the odds of being a PSA failure. For pERK, which ranged from 0 to 300, a 100-point decrease in staining score would result in an 80% increase in the odds of being a PSA failure. Each of these effects assumes the other biomarker is held constant. The interaction of pAkt and pERK was not significant (P > 0.40), indicating that their effects are independent.

To determine discrimination, the sensitivity and specificity of the given data were identified (14). The sensitivity of a test is defined as the true positive rate (disease present when the test is positive), whereas the specificity of a test is defined as the true negative rate (disease absent when test is negative). The area under the receiver operating characteristic (ROC) curve was determined from the plot of sensitivity versus (1 – specificity) [true positive rate versus false positive rate] and is a measure of the predictability of a test (Fig. 2B). An area under the ROC curve of 0.8–0.9 is considered excellent discrimination, whereas a ROC value of 0.5 indicates no discrimination (14). The Hosmer-Lemeshow goodness of fit test indicates a good fit for pAkt as well as pAkt and pERK combined. The ROC area for pAkt alone was 0.806 (P = 0.0008), and that for pERK alone was 0.615 (P = 0.34); however, the combined ROC area for these two biomarkers predicting PSA failure is 0.84 (P = 0.0018), indicating excellent discrimination (Fig. 2B). Thus pAkt, alone or together with pERK, is an excellent discriminator of PSA failure versus non-failure, whereas pERK by itself is not. The point T of optimum sensitivity and specificity (Fig. 2B) corresponds with a cut point of 0.78 in Fig. 2A (dashed line); all combinations of pAkt and pERK above the cutoff are considered as predictive of PSA failure.

Ki67 Is Not an Important Predictor of PSA Failure. The cell proliferation antigen Ki67 has often been shown to be an important predictor of poor clinical outcome (15). Nuclear staining for Ki67 is indicative of proliferation and quantitated as the percentage of cells staining for Ki67 (Ki67 LI). Table 2 summarizes the Ki67 LI data for...
55 available cases. In general, cases showing high Ki67 LI were PSA failures. The mean Ki67 LI in our cases was 16.12/14.97 (n = 37) for PSA failure and 9.04/10.52 (n = 18) for non-failure cases. Ki67 LI alone, when log-transformed, was also an acceptable predictor of PSA failure (area under the ROC curve = 0.67; P < 0.09) but was not as good a discriminator as pAkt. Ki67 LI did not add significantly to prediction of PSA failure once the effect of pAkt and pERK was considered.

Gleason Grading Is Not a Significant Predictor of PSA Failure. Often, Gleason grading is used as a predictor of clinical outcome (16). We therefore also determined whether Gleason score at the time of the prostatectomy predicted PSA failure in the same cases (Table 3). Gleason score at prostatectomy was not significant in predicting PSA failure (6.34 ± 1.88; n = 38) versus non-failure (5.75 ± 1.80; n = 20). Gleason score was also not a significant discriminator (P > 0.25) for PSA failure versus non-failure with an area under the ROC curve of 0.563.

**DISCUSSION**

In this study, we show that increased pAkt, alone or together with decreased pERK, is an excellent predictor of poor clinical outcome when PSA failure is used as the surrogate for poor clinical outcome.

<table>
<thead>
<tr>
<th>Intensity score (pAkt or pERK)</th>
<th>Frequency (%) of patients exhibiting pAkt staining in the indicated range</th>
<th>Frequency (%) of patients exhibiting pERK staining in the indicated range</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>PSA failure</td>
</tr>
<tr>
<td>0</td>
<td>4</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>1–100</td>
<td>8</td>
<td>4 (50%)</td>
</tr>
<tr>
<td>101–200</td>
<td>18</td>
<td>12 (66.67%)</td>
</tr>
<tr>
<td>201–300</td>
<td>23</td>
<td>21 (91.3%)</td>
</tr>
<tr>
<td>Total</td>
<td>53</td>
<td>37</td>
</tr>
</tbody>
</table>

*pAkt, phosphorylated Akt; pERK, phosphorylated extracellular signal-regulated kinase; PSA, prostate-specific antigen.*
In contrast to Akt, commonly used diagnostic methods, such as Gleason grading, did not predict clinical outcome. The cell proliferation antigen Ki67, which is commonly used as an indicator of proliferation, is also a good predictor of PSA failure, although it is not as good as pAkt. This is a significant result because current diagnostic procedures cannot distinguish between clinically aggressive and clinically indolent prostate cancer, resulting in more men being treated for the disease than necessary (4).

The observation that the active form of Akt may be a predictor of clinical outcome may be due to the apparent role this protein plays in prostate cancer progression. Akt is a downstream effector of the phosphatidylinositol 3'-kinase pathway, which was determined to be the dominant growth factor-activated cell survival pathway in the androgen-dependent LNCaP prostate tumor cell line (17). Akt activation was markedly increased in an androgen-independent LNCaP cell line isolated from LNCaP xenografts, compared with the parental androgen-dependent cell line (18). Expression of constitutively active Akt in androgen-dependent LNCaP cells resulted in a 6-fold increase in xenograft tumor growth (18). Androgen deprivation in androgen-dependent LNCaP human prostate cancer cells stimulated the activation of Akt, which eventually resulted in androgen independence of the cells (19). We and others (10, 11, 20) have shown significantly increased activation (phosphorylation) of Akt in high-Gleason grade prostate cancers. Our present results show the importance of Akt activation (phosphorylation) in prostate cancer progression to androgen independence and poor clinical outcome.

ERK is a member of the mitogen-activated protein kinase family of protein kinases. Like Akt, ERK is also stimulated by growth factors and cytokines. ERK has been implicated in the regulation of cell proliferation and differentiation; hence, we expected that the activation levels of ERK would also increase in aggressive prostate cancer or androgen-independent prostate cancer. Instead, poorly differentiated prostate cancers (10), as well as prostate cancers that have poor clinical outcome, express decreased levels of pERK (indicative of inactivation) compared with well-differentiated cancers and cancers with good clinical outcome, respectively. This suggests that ERK is not associated with cell proliferation or survival in human prostate cancer, similar to our previous observations in TRAMP mouse prostate cancer cells (21). Although others report a 15-fold increase in

![Graph A](image)

**Fig. 2. A,** predicted probability of PSA failure for different pAkt and pERK staining intensities. For each value of pERK (0–300), the predicted probability of PSA failure was calculated by logistic regression analysis and plotted against different pAkt staining intensities. This figure indicates that for a given value of pERK staining, the predicted probability of PSA failure increases with pAkt staining. The optimum predicted probability of 0.78 (dashed line) represents 80% sensitivity and 78% specificity. Any combination of pERK and pAkt with a higher predicted probability of PSA failure (points above the dashed line) represents a bad prognosis. For example, a pERK staining score of 150 requires a high pAkt staining score (>230) for the predicted probability of PSA failure to be 0.78 or higher. On the other hand, if the staining score for pERK was 50, a pAkt staining score of 200 or more would be needed for a similar predicted probability of PSA failure. The Hosmer-Lemeshow goodness of fit test indicates a good fit for pAkt and pERK combined (P > 0.8). B, plot of sensitivity versus specificity. An area under the ROC curve of 0.7–0.9, calculated from these values, is considered excellent discrimination (14). Combination of pAkt and pERK gives an area under the ROC curve of 0.84, indicating excellent discrimination. The optimum point \( T = (0.22, 0.8) \) represents the point of maximum sensitivity (closest to 1) for a minimum value of \( (1 - \text{specificity}) \) and corresponds to the cut point of 0.78 in A.

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**Table 2**

Ki67 LI\(^a\) scores in PSA failure and PSA non-failure

For each tumor, 500 cells were examined for nuclear staining for Ki67, and the mean percentage of cells expressing Ki67 (Ki67 LI) was calculated. The tissues were then classified according to Ki67 LI values.

<table>
<thead>
<tr>
<th>Ki67 LI</th>
<th>n</th>
<th>PSA failure</th>
<th>PSA non-failure</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>6</td>
<td>4 (66.67%)</td>
<td>2 (33.33%)</td>
</tr>
<tr>
<td>1–4.9</td>
<td>14</td>
<td>6 (42.9%)</td>
<td>8 (57.1%)</td>
</tr>
<tr>
<td>5–9.9</td>
<td>5</td>
<td>4 (80%)</td>
<td>1 (20%)</td>
</tr>
<tr>
<td>10–14.9</td>
<td>11</td>
<td>7 (63.6%)</td>
<td>4 (36.4%)</td>
</tr>
<tr>
<td>15–19.9</td>
<td>10</td>
<td>9 (90%)</td>
<td>1 (10%)</td>
</tr>
<tr>
<td>≥20</td>
<td>9</td>
<td>7 (77.8%)</td>
<td>2 (22.2%)</td>
</tr>
<tr>
<td>Total</td>
<td>55</td>
<td>37</td>
<td>18</td>
</tr>
</tbody>
</table>

\(^a\) LI, labeling index; PSA, prostate-specific antigen.

**Table 3**

Gleason scores in PSA\(^a\) failure and non-failure

The specimens described in Tables 1 and 2 are graded according to Gleason score in the table below:

<table>
<thead>
<tr>
<th>Gleason score</th>
<th>n</th>
<th>PSA failure</th>
<th>PSA non-failure</th>
</tr>
</thead>
<tbody>
<tr>
<td>2–4</td>
<td>9 (100%)</td>
<td>5 (55.56%)</td>
<td>4 (44.44%)</td>
</tr>
<tr>
<td>5–6</td>
<td>22 (100%)</td>
<td>15 (68.18%)</td>
<td>7 (31.82%)</td>
</tr>
<tr>
<td>7–10</td>
<td>27 (100%)</td>
<td>18 (66.67%)</td>
<td>9 (33.33%)</td>
</tr>
</tbody>
</table>

\(^a\) PSA, prostate-specific antigen.
ERK1/2 activation in prostatic intraepithelial neoplasia compared with normal human prostate tissue (22), we see a much smaller increase from normal tissue to prostatic intraepithelial neoplasia, which is thought to represent the precursor of prostate cancer (10).

Hence, it is likely that proliferation and survival are mediated by the phosphatidylinositol 3'-kinase/Akt pathway at all stages of prostate cancer development. Similar to Paweletz et al. (11), we observe a steady decline in pERK expression from prostatic intraepithelial neoplasia to poorly differentiated prostate cancer (10). Activation of ERK stimulated neuroendocrine differentiation in LNCaP cells (23). This suggests that ERK mediates differentiation in the prostate. Thus, decreased pERK alone may not be as good a predictor of PSA failure as elevated pAkt levels.

The cell proliferation antigen Ki67 was another good predictor of PSA failure, although it was not as strong as pAkt. Any indicator of proliferation may be expected to be a good predictor of clinical outcome because aggressive prostate cancers would have a higher proliferation rate compared with indolent tumors. However, the rate of proliferation alone does not determine the rate of tumor growth. In the normal adult prostate, the rate of proliferation is balanced by an equal rate of apoptosis. In prostate cancer, there is either increased proliferation, decreased apoptosis, or both, so that the cell number increases. Akt is a well-known regulator of cell survival, with significant antiapoptotic activities. Activation of Akt has been implicated in protection from apoptosis in response to growth factors, cytokines, c-myc overexpression, and matrix detachment (24, 25). Akt promotes cell survival by phosphorylating and inactivating the proapoptotic protein BAD (24). In addition, Akt stimulates cell cycle progression by phosphorylating glycogen synthase kinase 3, which stimulates cyclin D1 transcription, and the AFX/Forkhead family of transcription factors (26, 27), which suppresses AFX-mediated transcription of target genes such as the cyclin-dependent kinase inhibitor p27Kip1 (28). Thus, activation of Akt regulates both cell survival and proliferation and hence would be expected to be a better predictor of aggressive prostate cancer than Ki67 alone. Finally, we show that Gleason grading, which is often used to predict clinical outcome, was not a good predictor of PSA failure in our study. This may be due to the fact that only nine of our cases had low Gleason scores (Gleason scores of 2–4); perhaps an even distribution of high and low Gleason scores would have yielded results in which Gleason grading played a more significant role in predicting PSA failure.

In conclusion, we have shown that increased pAkt, alone or together with decreased pERK, is an important predictor of the probability of PSA failure. Phosphorylation of these proteins may therefore be considered a useful biological marker of clinically aggressive cancer. Replication of these results and the inclusion of all known prognostic factors in the study would strengthen the utility of these biomarkers alone and in combination. A replicated study that uniformly and accurately measures time to PSA failure would also strengthen the results.

REFERENCES

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