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Suppression of 19S proteasome subunits marks emergence of an altered cell state in diverse cancers

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The use of proteasome inhibitors to target cancer’s dependence on altered protein homeostasis has been greatly limited by intrinsic and acquired resistance. Analyzing data from thousands of cancer lines and tumors, we find that those with suppressed expression of one or more 19S proteasome subunits show intrinsic proteasome inhibitor resistance. Moreover, such proteasome subunit suppression is associated with poor outcome in myeloma patients, where proteasome inhibitors are a mainstay of treatment. Beyond conferring resistance to proteasome inhibitors, proteasome subunit suppression also serves as a sentinel of a more global remodeling of the transcriptome. This remodeling produces a distinct gene signature and new vulnerabilities to the proapoptotic drug, ABT-263. This frequent, naturally arising imbalance in 19S regulatory complex composition is achieved through a variety of mechanisms, including DNA methylation, and marks the emergence of a heritably altered and therapeutically relevant state in diverse cancers.

Cancer cells rely on the proteasome machinery to mediate protein turnover and maintain protein homeostasis (1–3). Upon oncogenic transformation, a myriad of proteotoxic stresses that tax the cellular machinery responsible for protein homeostasis are introduced (2, 4). These pressures cause cancer cells to rely heavily on enhanced proteasome function (2, 5–8). This dependency can be exploited using natural and synthetic compounds that inhibit, with exquisite potency and selectivity, the catalytic function of the 20S proteasome (9, 10). Indeed, these compounds are highly effective at inhibiting the growth of a wide variety of cancer cell lines in culture. Unfortunately, their clinical utility has been surprisingly limited, with therapeutic benefits commonly observed for only a few types of cancer (9, 10).

The limited role for proteasome inhibitors as clinical chemotherapeutics can be attributed to biological processes that promote intrinsic and acquired resistance. For example, cell culture models of acquired resistance often accumulate mutations in the catalytic subunits of the 20S proteasome (11, 12). In terms of clinical relevance, however, such mutations have yet to be detected in clinical samples (13). Additional mechanisms of resistance include alteration of specific cellular pathways such as constitutive activation of NF-κB (14), activation of the chaperone machinery (15, 16), or alterations in the EGFR/JAK1/STAT3 pathway (17).

To further explore and identify the cellular mechanisms of resistance developed in the presence of distinct proteasome inhibitors, our group and others recently applied genome-wide genetic screens to various cell-line models (18, 19). In all models examined, experimentally reducing the expression of one of the many different subunits composing the 19S regulatory complex increases resistance to inhibitors of the proteasome’s catalytic core (18, 19) and may show clinical relevance in multiple myeloma patients (18). These data suggested that transient, nonmutational mechanisms may play a key role in the ability of cancer cells to withstand proteasome inhibition. In this work we examine if such a mechanism is implemented by different cancers.

Results

Sharply Reduced Expression of One 19S Proteasome Complex Subunit Occurs Frequently Across Diverse Cancer Cell Lines. We first characterized relative mRNA expression levels for all genes that encode proteasome subunits using the Genomics of Drug Sensitivity in Cancer (GDSC) database (20). As expected, across 789 cancer cell lines, the average expression level of 20S subunits was highly correlated with the average expression level of 19S subunits (Fig. 14). However, in some cell lines, the expression of a specific 19S subunit was much lower than the average expression of that subunit across all cell lines. To quantify this phenomenon for each cell line in the GDSC and the Cancer Cell Line Encyclopedia (CCLE) datasets, we calculated the SD from the mean of every 19S subunit in every cell line. Each cell line was then assigned a sigma score that is indicative of the greatest deviation from the mean of any one 19S subunit in that cell line. Thus, a sigma score of 3 indicates a cell line in which the mRNA expression of at least one subunit of the 19S proteasome complex was reduced by 3 SDs from the mean mRNA level of that particular subunit across all cell lines. A sigma score of 2 indicates a reduction in the mRNA of 2 SDs, and so on.

In 59 cancer cell lines, the mRNA level of at least 19S subunit 3 or more SDs (3σ) from the mean for that subunit.

Significance

In previous work, we used genome-wide screening to uncover a counterintuitive mechanism by which cells can acquire resistance to inhibitors of the proteasome’s catalytic core through experimentally induced imbalances in the composition of its regulatory particle. However, in many cases, mechanisms uncovered in vitro for acquired resistance often do not translate to the context of actual clinical cancers. Here, we show that this mechanism is actually deployed spontaneously and naturally in diverse human cancer lines and is associated not only with increased resistance to proteasome inhibitors both in vitro and in the clinic but also is symptomatic of a much more broadly altered state with a unique gene signature and drug targetable vulnerabilities.

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Reduced expression of a single 19S proteasome complex subunit occurs naturally in many cancer cells. (A) The overall expression of all 26S proteasome subunits is tightly correlated. The correlation between the z-score sum of expression of 20S proteasome subunits versus the 19S proteasome subunits is plotted across 789 cancer cell lines taken from the GDSC dataset. Spearman correlation: $r = 0.84$ ($P < 0.0001$). (B and C) Representation of the expression levels for individual 19S subunits for each cancer cell line in the GDSC (B) and the CCLE datasets (C). Red dots indicate cells that exhibit reduced expression of a given subunit by more than 3 SDs from the mean (3σ). Each dot represents an individual cell line. (D) Analysis of primary tumor expression profiles taken from TCGA dataset. The value of each bar is the number of primary tumors exhibiting a 3σ drop in expression of at least one 19S subunit.

Fig. 1. Reduced expression of any one of many 19S subunits correlates highly with resistance to proteasome inhibitors. We next examined whether naturally occurring reduced expression of a 19S subunit correlated with the cells’ ability to tolerate proteasome inhibition. We used 310 cell lines in the GDSC collection for which drug-sensitivity data were available and asked if our calculated sigma score correlated with the measured EC50 for two chemically distinct proteasome inhibitors, bortezomib and MG132. The correlation between sigma score and increased EC50 with increased resistance to both bortezomib and MG132. The correlation between sigma score and increased EC50 for proteasome inhibitors plateaued at approximately a sigma score of 3; cells with sigma scores of 4 or 5 did not have significantly higher EC50’s than cells with sigma scores of 3 (Fig. 2A and Fig. S2A). Increases in the sigma score (i.e., reduced expression of a single 19S subunit) were highly correlated with increased resistance to both bortezomib and MG132. The correlation between sigma score and increased EC50 for proteasome inhibitors plateaued at approximately a sigma score of 3; cells with sigma scores of 4 or 5 did not have significantly higher EC50’s than cells with sigma scores of 3 (Fig. 2A and Fig. S2A). Comparisons of the mean EC50 for both bortezomib and MG132 between the control group of cell lines and the 3σ lines revealed a significantly higher EC50 in the 3σ group ($P < 0.0001$) (Fig. 2B and Fig. S2B). For bortezomib, the average natural log of the EC50 of the control group was −5.68, whereas the average natural log of the EC50 of the 3σ group was −3.56 (Fig. 2B). A similar sigmascore analysis for 20S proteasome subunit expression did not
Reduced 19S Subunit mRNA Expression Correlates with Inhibitor Resistance and Poor Outcome for Myeloma Patients Treated with Bortezomib. To determine whether the subunit-suppression mechanism of resistance that we identified has relevance in therapeutic settings, we analyzed gene-expression data from two sources: a laboratory-generated model of acquired resistance to proteasome inhibition (23) and patient tumor samples collected during clinical trials of bortezomib (22). The laboratory model involved the mantle cell lymphoma line Jeko-1 and a bortezomib-resistant variant (JBR cells) (23). The transcriptional profile of xenografts formed by these cell lines in mice (21) revealed a strong reduction in PSMDS5 mRNA levels in the tumors formed by the bortezomib-resistant JBR cell lines. For patient-derived data, we mined correlative studies performed as part of key phase 2 and 3 clinical trials that were conducted in 2007 and established the efficacy of bortezomib in patients with recurrent multiple myeloma (22). Expression profiling data for pretreatment myeloma samples were available from these trials but posttreatment samples were not collected, precluding analysis of 19S subunit levels in the myeloma cells that emerged following bortezomib treatment and relapse. Strikingly, even in samples obtained before treatment, we found that reduced 19S subunit expression at baseline correlated with inferior disease control by bortezomib (Fig. 2F). Samples from 54 of 264 bortezomib-naïve patients exhibited reduced expression of at least one of the 19S proteasome subunits. Of these 54 cases, 34 patients subsequently received bortezomib. These 34 patients exhibited a significantly shorter time to progression compared with the patients with myeloma that did not have relative suppression of 19S subunit expression (p = 0.0001) (Fig. 2F). Notably, bortezomib treatment of patients with reduced expression of 19S proteasome subunit(s) showed no greater effectiveness than dexamethasone treatment in the control group. Nor did suppression of 19S subunit(s) induce a significant change in the efficacy of dexamethasone treatment (Fig. S2C).

Proteasome 19S Subunit Suppression Marks a Distinct Heritable State. To examine if the altered proteasome inhibitor sensitivity in the cells with 19S subunit suppression is part of a broader alteration to the cellular state, we created a rank-ordered list of gene-expression alterations in the 3r cell lines. Analysis of CCLE and the GDSC datasets, comprising more than 1,000 cell lines, yielded very similar results. Cells with reduced expression of 19S subunits had a significant reduction in genes associated with several key oncogenic and signaling pathways. For example, the epithelial–mesenchymal transition (EMT) gene signature was significantly reduced (Fig. 3A, yellow, and Fig. S3 A and B). These cells also displayed a reduction in gene signatures related to EGF and TNF signaling (Fig. 3C, green, and Fig. S3A) and heat-shock–related genes (Fig. 3C). Such highly significant patterns of differential gene expression and gene-set enrichment are compelling, particularly given that they are derived from many diverse cancer cell lines. Given the strong suppression of an EMT signature in the cell lines, we focused additional analyses on the expression profiles of primary breast cancer samples, a tumor type in which EMT
plays a critical role in tumor progression, in invasion, and, ultimately, in systemic metastasis (24, 25). We used mRNA expression data from the TCGA and defined breast tumors as a “control” unless they had a 3σ reduction of at least one subunit of the 19S proteasome. The genes that were specifically enriched in the proteasome 3σ tumors were primarily related to mitochondrial oxidative phosphorylation and to ribosomal biogenesis (Fig. 3B, red, and Fig. S3D). Such findings suggest that the 3σ tumors might have undergone a less complete shift to aerobic glycolysis (“Warburg effect”) and therefore may have retained higher dependency on mitochondrial respiration than the control tumors.

Gene-set enrichment analysis further revealed that the unique signature of the 3σ cancers is conserved between cancer cell lines and primary tumors. For example, the EMT gene signature was largely suppressed in 3σ primary breast tumors (Fig. S3D). Extracellular matrix (ECM) genes were also suppressed in the 3σ tumors; ECM genes are highly relevant to both EMT and EGFR signaling. Thus, in both established tumor cell lines and in primary tumors, cells with a 3σ reduction in any one 19S proteasome subunit are similar in many other malignancy-related biological pathways.

Naturally Occurring 19S Subunit Suppression Confers Vulnerability to ABT-263. Our data indicate that single-supubit imbalances in the 19S regulatory complex are part of a broad shift in the status of many cancer cells. Using publicly available drug-screening datasets, we asked if this naturally occurring, altered state might change the sensitivity of diverse cancer lines to drugs in addition to proteasome inhibitors. We examined sensitivity data for 140 drugs, most of which were screened against 655 cell lines from the GDSC dataset. For each drug, we calculated the average EC50, which were screened against 655 cell lines from the GDSC dataset. For each drug analyzed. P values were calculated by the student’s t test. (D) The Mann–Whitney test was used to calculate a P value for ABT-263 average sensitivity comparing the 3σ and the non 3σ cells in the GDSC dataset. (E) Relative viability was examined in IMR32 (natural suppression of PSMD5) and Kelly cells 72 h after addition of the indicated concentrations of ABT-263.

Mechanisms That Reduce Expression of Specific 19S Proteasome Subunits in Cancers. Rewiring of the transcriptome can result from either genomic aberrations or epigenomic changes. Many chromosomal regions are recurrently lost in cancers, and some of these regions harbor genes that encode 19S proteasome subunits, such as PSMC2 (26). DNA sequencing data from the CCLE resource enabled us to determine whether the differential reduction in 19S subunit mRNA expression was associated with copy-number loss. Notably, in the majority of 3σ cell lines, the reduced mRNA expression of 19S subunits was not associated with gene-copy-number losses (Fig. 4A). For example, in the cases of PSMC3, PSMC4, PSMD3, PSMD5, PSMD7, PSMD8, and PSMD10 subunits, the 3σ lines did not exhibit any copy-number losses involving these genes. Other 19S 3σ subunit lines exhibited partial gene-copy-number losses (Fig. 4A). Thus, a mechanism other than gene-copy-number alterations is largely responsible for the reduction of 19S subunit expression in most cancer cell lines.

To investigate further, we examined regulation of PSMD5 as it was the most frequently suppressed 19S subunit across cancer cell lines and tumors (Fig. 1). A common mechanism suppressing the expression of genes is methylation of their promoters. We assessed PSMD5 promoter methylation in both low-grade gliomas
Suppression of the 19S subunit PSMD5 in a variety of cancers is an epigenetic process regulated by DNA promoter methylation. (A) The number of 3σ events versus the number of gene copy loss for every 19S subunit. A cell line was considered to have a copy-number loss if the estimated hybridization was 0.5x the baseline hybridization. (B) TCGA dataset analysis for PSMD5 promoter methylation in LGG primary tumors. The data are aligned according to expression levels of the PSMD5 gene (on the left). The methylation score was analyzed for probes I–IV corresponding to genomic coordinates 123605229, 123605234, 123605306, and 123605570, respectively. (C and D) The average methylation score was calculated for the PSMD5 promoter region in LGG (C) and BLCA (D) primary tumors. Scores were calculated separately for tumors with a sigma score >3 for the PSMD5 gene (3σ) and the remainder (control). The mean and SEM for each indicated probe is plotted according to the corresponding genomic coordinates. The methylation score was analyzed for probes I–IV corresponding to the same genomic coordinates as in B. ***p < 1e-5 (t test). (E) The mRNA levels and the methylation score for each cell line from the CCLE dataset are plotted for the PSMD5 gene. Annotated cell lines that have reduced expression and a methylated PSMD5 promoter are marked in red. (F) The frequency of methylated CpGs in the promoter region of the PSMD5 gene in IMR32 and Kelly cells.

Fig. 4. Suppression of the 19S subunit PSMD5 in a variety of cancers is an epigenetic process regulated by DNA promoter methylation. (A) The number of 3σ events versus the number of gene copy loss for every 19S subunit. A cell line was considered to have a copy-number loss if the estimated hybridization was 0.5x the baseline hybridization. (B) TCGA dataset analysis for PSMD5 promoter methylation in LGG primary tumors. The data are aligned according to expression levels of the PSMD5 gene (on the left). The methylation score was analyzed for probes I–IV corresponding to genomic coordinates 123605229, 123605234, 123605306, and 123605570, respectively. (C and D) The average methylation score was calculated for the PSMD5 promoter region in LGG (C) and BLCA (D) primary tumors. Scores were calculated separately for tumors with a sigma score >3 for the PSMD5 gene (3σ) and the remainder (control). The mean and SEM for each indicated probe is plotted according to the corresponding genomic coordinates. The methylation score was analyzed for probes I–IV corresponding to the same genomic coordinates as in B. ***p < 1e-5 (t test). (E) The mRNA levels and the methylation score for each cell line from the CCLE dataset are plotted for the PSMD5 gene. Annotated cell lines that have reduced expression and a methylated PSMD5 promoter are marked in red. (F) The frequency of methylated CpGs in the promoter region of the PSMD5 gene in IMR32 and Kelly cells.

Discussion

The transcriptional program that regulates proteasome subunit mRNA expression is highly coordinated to maintain the stoichiometric balance of the multiple proteasome components and to foster the efficient assembly of the 26S proteasome complex (27–29). However, a significant change in proteasome complex assembly can result as a consequence of alterations in the level of expression of just a single subunit (26, 30–33). Examining thousands of cancer lines, we show that imbalanced expression of the proteasome subunits composing the 19S regulatory complex occurs through a variety of mechanisms. In the case of the PSMD5 subunit, multiple cancers displayed suppression mediated by promoter DNA methylation. However, other subunits did not use this mechanism. Indeed, other laboratories have shown that several types of cancer display chromosomal loss of regions encompassing the locus encoding the PSMD2 subunit. This deletion has a twofold effect: a decrease both in overall 26S proteasome levels and in the ratio of 26S to 20S proteasomes (26). Moreover, reduced expression of PSMD2 has been associated with poor response of multiple myeloma patients to carfilzomib treatment (18). Thus, alterations in both DNA methylation and gene copy number are clearly involved in regulating the expression of 19S subunits in a wide variety of cancers. Additional mechanisms, including histone modifications, new mutations, and microRNAs, are also likely to be deployed.

Regardless of the mechanism of suppression, however, the reduction in any one of the many subunits composing the 19S complex reflects a largely altered cellular state characterized by increased resistance to proteasome inhibitors, altered gene-expression signatures, and increased sensitivity to the BCL2 family inhibitor ABT-263 and a small cohort of other clinically relevant drugs. For example, the TNF-NFκB pathway is suppressed in cells that exhibit reduced expression of at least one 19S proteasome subunit. This down-regulation is consistent with the observation that proteasome inhibitors have a strong inhibitory effect on NFκB activation (34). Furthermore, the TNF→NFκB pathway regulates the transcription of specific proteasome subunits (including PSMD5) (35). Perhaps the most striking difference observed in the 3σ group, however, is the altered expression of genes that are associated with the process of EMT. Cell lines in which expression of a single 19S subunit is suppressed display increased epithelial characteristics indicative of a less invasive, less mesenchymal state. Strikingly, in KRAS mutant cell lines an enhanced epithelial gene signature correlates with increased sensitivity to ABT-263 (36),
consistent with our findings here. Moreover, analysis of primary breast cancers revealed that 36 tumors display reduced aerobic glycolysis and higher dependency on oxidative phosphorylation/ mitochondrial respiration. At a functional level, it is tempting to speculate that the hypersensitivity to ABT-263, which would be expected associated with imbalanced 19S subunit expression, is somehow linked to the alterations in mitochondrial physiology suggested by our gene-set enrichment analysis.

Our findings imply that some tumors—or, perhaps equally important, some cancer cells within a heterogeneous tumor—exist in an altered cellular state in which 19S subunit expression is imbalanced. This state appears to be part of a larger cell program that extends beyond the regulation of proteasome function and includes metabolism and signaling. Furthermore, although the more epithelial, more differentiated state marked by regulatory subunit imbalance may be disfavored in the sole context of increased proliferation, it could well serve as a bet-hedging mechanism within the overall tumor to enhance the survival of cells in which protein homeostasis is particularly challenged. Devising orthogonal strategies for targeting this and the other varied epigenetic and phenotypic states available to tumor cells over the course of malignant progression may serve to limit adaptations to chemotherapy, block the emergence of drug-resistant clones, and increase our ability to eradicate advanced cancers.

Materials and Methods

Dataset Analysis. Gene expression and drug sensitivity data were downloaded from www.cancergenome.org/ (GDSC), https://www.broadinstitute.org/ (CCLE), and https://cancergenome.nih.gov/ (TCGA). For each proteasome subunit gene, a z-score was calculated for each cell line and that was used to plot the expression of individual subunits across all cells in the dataset (see SI Materials and Methods for details). EC50s for the different drugs were extracted from the GDSC dataset and analyzed as described (see SI Materials and Methods for details). Gene copy estimates were extracted and calculated from the CCLE dataset. PSMSD promoter methylation scores were extracted from both the CCLE and TCGA datasets (see SI Materials and Methods for details).

DNA Methylation Sequencing. DNA was extracted from IMR32 and Kelly cells with a DNeasy Blood and Tissue Kit (Qiagen) according to the manufacturer’s protocol. Bisulfate conversion of the DNA was conducted using the EpiTect Bisulfite Kit (Qiagen) according to the manufacturer’s protocol. The modified DNA was then used as a template for PCR of the PSMD5 promoter (see SI Materials and Methods for details).

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