RESEARCH BRIEF

Autophagy Is Critical for Pancreatic Tumor Growth and Progression in Tumors with p53 Alterations

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ABSTRACT

Pancreatic ductal adenocarcinoma is refractory to available therapies. We have previously shown that these tumors have elevated autophagy and that inhibition of autophagy leads to decreased tumor growth. Using an autochthonous model of pancreatic cancer driven by oncogenic Kras and the stochastic LOH of Trp53, we demonstrate that although genetic ablation of autophagy in the pancreas leads to increased tumor initiation, these premalignant lesions are impaired in their ability to progress to invasive cancer, leading to prolonged survival. In addition, mouse pancreatic cancer cell lines with differing p53 status are all sensitive to pharmacologic and genetic inhibition of autophagy. Finally, a mouse preclinical trial using cohorts of genetically characterized patient-derived xenografts treated with hydroxychloroquine showed responses across the collection of tumors. Together, our data support the critical role of autophagy in pancreatic cancer and show that inhibition of autophagy may have clinical utility in the treatment of these cancers, independent of p53 status.

SIGNIFICANCE: Recently, a mouse model with embryonic homozygous Trp53 deletion showed paradoxical effects of autophagy inhibition. We used a mouse model with Trp53 LOH (similar to human tumors), tumor cell lines, and patient-derived xenografts to show that p53 status does not affect response to autophagy inhibition. These findings have important implications on ongoing clinical trials. Cancer Discov; 4(8): 905–13. © 2014 AACR. See related commentary by Amaravadi and Debnath, p. 873.

INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC) accounts for 95% of pancreatic cancers and is one of the most lethal cancers, with a 5-year survival rate of approximately 6% (1). More than 80% of patients are diagnosed at an advanced stage, and treatment is very limited. Previously, we identified that human PDACs have elevated basal autophagy, and either genetic or pharmacologic inhibition of autophagy [the latter with chloroquine (CQ) treatment] showed robust...
As a result, a conditional Atg5 deletion allele or a Trp53 deletion allele or a TP53 deletion allele or a Trp53 deletion allele, as had been shown previously with Atg7 deletion in that compartment (ref. 21; Supplementary Fig. S1A and S1B). Thus, Atg5L−/− mice had a reduced long-term survival that was consistent with previous reports (Fig. 1B; ref. 19). Given the relatively restricted phenotype of the Atg5L−/− mice and the long survival of this cohort, we crossed Atg5L−/− mice into the PDAC GEMM (Pdx1Cre; iIdKrasG12D/+; Trp53L−/−) to assess the role of autophagy in PDAC development. To this end, we generated three cohorts with differing Atg5 allelic status: Atg5+/+, Atg5L−/−, and Atg5S−/−. This GEMM recapitulates the human condition beginning from premalignant pancreatic intraepithelial neoplasia (PanIN) to invasive and malignant PDAC (16, 18). We first compared the overall survival between the groups and found that the Atg5S−/− cohort had the longest median survival (Fig. 1C). Indeed, there were significantly more long-term survivors (>30 weeks of age) in this group compared with either the Atg5L−/− or Atg5S−/− cohorts (P = 0.0005). Interestingly, we observed that approximately 20% of the Atg5L−/− mice died before 6 weeks of age, which was not seen in the Atg5S−/− cohort (0%) and infrequently (4%) in the Atg5+/+ group. Analysis of this small population of Atg5L−/− mice revealed that they did not die of PDAC but due to acinar destruction as shown by massive acinar to ductal metaplasia, which led to the disruption of most of the exocrine pancreas and left on average less than 10% normal pancreas (Supplementary Fig. S2A and S2B). The majority of the Atg5S−/− cohort survived past this early insult and when death due to PDAC was assessed, the Atg5L−/− mice had a significantly prolonged survival compared with the Atg5+/+ and Atg5S−/− cohorts (P = 0.0032; Fig. 1D). To investigate the mechanism behind the prolonged survival in the Atg5S−/− mice, we performed a detailed histologic assessment of pancreata at an early time point (6 weeks) and a later time point (15 weeks) to determine how Atg5 loss affects both tumor initiation and progression. At 6 weeks, there was a marked difference in formation of noninvasive precursor lesions, PanIN, between the cohorts, with the Atg5+/+ group having on average more than 50 PanINs per mouse compared with the Atg5S−/− group, which showed only 2 to 5 PanINs per mouse (Fig. 1E). The difference in PanIN formation was further quantified by comparing the fraction of normal pancreas remaining with that containing PanIN and surrounding inflammation (22). The Atg5S−/− cohort showed 41.7% normal development. We also explored how tumor cell lines with TP53 deletions or mutations responded to autophagy inhibition. Finally, we determined how human patient-derived xenografts responded to autophagy inhibition using HCQ treatment to assess the clinical significance of TP53 genotypes on human tumor response.

RESULTS

To study the function of autophagy in pancreatic cancer development, we generated pancreatic conditional Atg5 knockout mice using a Pdx1 promoter–driven Cre recombinase (Fig. 1A; ref. 20). We first assessed the impact of homozygous deletion of Atg5 (Atg5L−/−) in the pancreas. Pancreatic deletion of Atg5 was not embryonic lethal and did not cause signs of malignant transformation; however, it caused a cellular disruption in endocrine tissues starting at 12 weeks, leading to a reduction in insulin-producing ß-cells, as had been shown previously with Atg7 deletion in that compartment (ref. 21; Supplementary Fig. S1A and S1B). Thus, Atg5L−/− mice had a reduced long-term survival that was consistent with previous reports (Fig. 1B; ref. 19). Given the relatively restricted phenotype of the Atg5L−/− mice and the long survival of this cohort, we crossed Atg5L−/− mice into the PDAC GEMM (Pdx1Cre; iIdKrasG12D/+; Trp53S−/−) to assess the role of autophagy in PDAC development. To this end, we generated three cohorts with differing Atg5 allelic status: Atg5+/+, Atg5L−/−, and Atg5S−/−. This GEMM recapitulates the human condition beginning from premalignant pancreatic intraepithelial neoplasia (PanIN) to invasive and malignant PDAC (16, 18). We first compared the overall survival between the groups and found that the Atg5S−/− cohort had the longest median survival (Fig. 1C). Indeed, there were significantly more long-term survivors (>30 weeks of age) in this group compared with either the Atg5L−/− or Atg5S−/− cohorts (P = 0.0005). Interestingly, we observed that approximately 20% of the Atg5L−/− mice died before 6 weeks of age, which was not seen in the Atg5S−/− cohort (0%) and infrequently (4%) in the Atg5+/+ group. Analysis of this small population of Atg5L−/− mice revealed that they did not die of PDAC but due to acinar destruction as shown by massive acinar to ductal metaplasia, which led to the disruption of most of the exocrine pancreas and left on average less than 10% normal pancreas (Supplementary Fig. S2A and S2B). The majority of the Atg5S−/− cohort survived past this early insult and when death due to PDAC was assessed, the Atg5L−/− mice had a significantly prolonged survival compared with the Atg5+/+ and Atg5S−/− cohorts (P = 0.0032; Fig. 1D). To investigate the mechanism behind the prolonged survival in the Atg5S−/− mice, we performed a detailed histologic assessment of pancreata at an early time point (6 weeks) and a later time point (15 weeks) to determine how Atg5 loss affects both tumor initiation and progression. At 6 weeks, there was a marked difference in formation of noninvasive precursor lesions, PanIN, between the cohorts, with the Atg5+/+ group having on average more than 50 PanINs per mouse compared with the Atg5S−/− group, which showed only 2 to 5 PanINs per mouse (Fig. 1E). The difference in PanIN formation was further quantified by comparing the fraction of normal pancreas remaining with that containing PanIN and surrounding inflammation (22). The Atg5S−/− cohort showed 41.7% normal...
pancreatic parenchyma versus 98.6% in the Atg5+/+ group (P = 0.0015; Fig. 1E). At 15 weeks, the normal pancreatic parenchyma in the Atg5−/− group further decreased to 31.7%, but interestingly only 25% of the mice (2 of 8) had invasive PDAC, whereas 60% of the Atg5+/+ mice (6 of 10) developed PDAC (Fig. 1E). Together, these data indicate that although autophagy deficiency increased PanIN development (tumor initiation), it inhibits PanIN progression to PDAC. IHC for ATG5 and LC3 confirmed that all tumors developed in Atg5−/− mice lacked ATG5 expression and had absent autophagy.
(Supplementary Fig. S3A and S3B). Interestingly, cell lines derived from two of the deleted tumors reestablished Atg5 expression after a few passages, indicating that rare subclones escaped Pdx1Cre-mediated excision and can grow out in a small fraction of the tumors (Supplementary Fig. S4A). As expected, tumors from all three genotypes showed LOH of the remaining Trp53 allele and did not express p53 protein (Supplementary Fig. S4B). We also assessed tumors from the three groups for the expression of cleaved caspase-3, γH2AX, and Ki67 (Fig. 1F) to measure apoptosis, DNA damage, and proliferation, respectively, and found that there was elevation of both apoptosis and DNA damage as well as a decrease in proliferation in the Atg5L/L group compared with the Atg5L/+ or Atg5L/L′ cohorts (Fig. 1F). Therefore, in the tumors that were able to form in the setting of autophagy loss, there was increased DNA damage and cell death. This is consistent with the fact that cell lines derived from these autophagy-incompetent tumors proliferate at a significantly slower rate than Atg5L/+ tumors under both high-serum and low-serum conditions (Fig. 1G).

Together, our data suggest that autophagy is required for proper progression of premalignant lesions to invasive PDAC, and those tumors that do progress are less robust.

Previously, we reported that human PDAC cell lines and tumors have elevated basal autophagy, and CQ treatment, as well as suppression of autophagy using RNAi against critical autophagy genes, could inhibit cell growth in vitro as well as in xenografts. In addition, CQ treatment significantly prolonged survival in the IslKrasG12D+/+; Trp53L/+ PDAC GEMM (2). A recent study using a Kras-driven PDAC GEMM with an embryonic Trp53 homozygous deletion showed that either Atg5 or Atg7 deletion promoted tumor progression, thus reducing mouse survival. In their model, CQ treatment seemed to have a modest reduction in lifespan as well. Given the differences between our prior and current data using the Trp53L/+ PDAC GEMM, and that reported by Rosenfeldt and colleagues (19) using the Trp53L/+ GEMM, we examined the impact of autophagy inhibition in a panel of murine PDAC cell lines with varying Trp53 genotypes (Trp53L/+; Trp53L/L, and p53R172H/-/-). As the majority of the human clinical trials are using CQ or its derivative, HCQ, we focused on response to CQ given its clinical relevance. As has been reported in the past (18), the wild-type (WT) allele of Trp53 was lost in Trp53L/+; Trp53L/L, and the Trp53R172H/-/- lines, as confirmed by PCR (Supplementary Fig. S5A). Western blot analysis showed loss of p53 expression in the Trp53L/+ and Trp53L/L lines, whereas Trp53 mutations were detected in the Trp53R172H/-/- lines (DNA point mutation confirmed by sequencing; Supplementary Fig. SSB and SSC). All PDAC lines, independent of the Trp53 genotype, showed a significant, dose-dependent reduction in clonogenic growth when treated with CQ (Fig. 2A). We had previously shown that one of the consequences of autophagy inhibition in human PDAC cells was a decrease in oxidative phosphorylation (measured by oxygen consumption; ref. 2). Consistent with these findings, all cell lines, independent of TP53 status, had a significantly reduced baseline oxygen consumption rate (OCR; Fig. 2B). To validate the CQ data, we repeated the clonogenic assays using shRNAs to either ATG5 or ATG7. Suppression of expression of both ATG genes and autophagy inhibition was confirmed by Western blot analysis (Supplementary Fig. S6). Similar to the CQ data, suppression of autophagy via RNAi significantly attenuated clonogenic growth independent of the TP53 genotype (Fig. 2C).

Finally, to model the therapeutic situation that is occurring in ongoing human clinical trials, we performed efficacy trials using 12 individual human patient-derived pancreatic cancer xenografts (PDX) treated with HCQ or saline control. Mice with established pancreatic tumors were treated with HCQ, and tumor growth was compared with the vehicle-treated group. The overwhelming majority of the PDX lines showed a reduction in tumor volume compared with controls (P < 0.05), with a third of the PDX lines showing more than 20% inhibition of tumor growth compared with the tumors in the vehicle-treated mice (Fig. 3B). All tumors had Kras mutations (except P410) and TP53 mutations (except JH024; Supplementary Table S1). Interestingly, consistent with previous findings of the role of autophagy in Kras mutant cancers (2, 8), the Kras WT tumor did not seem to have elevated autophagy by transmission electron microscopy (TEM) and did not respond to HCQ treatment (Fig. 3A and B). IHC for LC3 in the treated tumors showed that HCQ increased the LC3 punctate staining in the HCQ-treated samples, consistent with effective autophagy inhibition. In line with the TEM data, the Kras WT tumor had the lowest amount of basal puncta that did show an increase upon HCQ treatment (Fig. 3C). In addition, the fact that all the TP53 mutant tumors showed varying degrees of response further supports the fact that disruption of the p53 axis does not affect response to antiautophagy therapies. Ki67 and cleaved caspase-3 staining of the three best responders versus the Kras WT nonresponder was consistent with its effect on tumor volume: HCQ treatment significantly inhibited tumor cell proliferation and increased apoptosis in the responders but had minimal impact on the nonresponder (Fig. 3D and E).

DISCUSSION

In this study, we have used multiple orthogonal approaches (autotransplanted models, cell lines, and human tumor xenografts) to demonstrate that disruption of the p53 axis (a finding observed in 75% of PDAC) has no impact on the efficacy of autophagy inhibition. We used a PDAC GEMM (Pdx1Cre; IslKrasG12D+/+, Trp53L/+), in which Trp53 is lost by stochastic LOH as seen in human tumors and determined the role of autophagy in PDAC progression using a conditional Atg5 allele. We found that deletion of Atg5 predisposed mice to premalignant pancreatic lesions as evidenced by the increased occurrence of PanINs. On the other hand, mice with Atg5 deletion were significantly less likely to develop PDAC and therefore had improved survival.

The role of autophagy in tumorigenesis is controversial because there are studies supporting both its being a suppressor and a promoter. Evidence to support autophagy as a tumor suppressor comes from studies in which blocking autophagy genes were deleted in mice. With the exception of Becn1, in which the heterozygote was used and autophagy was only partially attenuated, these studies have shown that loss of autophagy predisposes mice to benign tumors (4, 23, 24). In contrast, evidence to support autophagy as a tumor promoter comes from studies of advanced tumors, in which blocking autophagy inhibits tumor growth and can synergize with
Autophagy Is Critical for Pancreatic Tumor Growth

Figure 2. Autophagy inhibition reduces colony formation and reduces baseline OCR independent of p53 status. A, CQ reduces clonogenic growth (blank bar, 0 μmol/L CQ; 10% dotted bar, 7.5 μmol/L CQ; 25% dotted bar, 15 μmol/L CQ). Error bars, SD of a representative experiment performed in triplicate. *, P < 0.005 by t test. B, CQ reduces baseline OCR in tumor cell lines. A representative OCR plot is shown for one cell line of each genotype. Error bars, SD of triplicate wells. *, P < 0.05 by t test. Bottom, quantification of the reduction in baseline OCRs from cell lines of each indicated Trp53 genotype. Error bars, SD from three experiments. C, clonogenic assay shows that knocking down autophagy reduces colony formation independent of Trp53 genotype. Blank bar, shGFP; 10% dotted bar, shAtg5; 25% dotted bar, shAtg7. Error bars, SD of a representative experiment performed in triplicate. *, P < 0.05 by t test.

Our data suggest a dual role for autophagy in PDAC development, whereby autophagy loss increases the initiation of tumors, but abrogates the efficient progression to invasive cancer.

Several prior studies have shown that autophagy deletion attenuates malignant tumor formation in lslKrasG12D/+ tumor models (8, 9). However, the role of p53 in this process is complex, with studies reporting results that differ in terms of how tumorigenesis affects autophagy loss with concurrent Trp53 deletion (9, 19). A recent study using a PDAC GEMM showed that the impact of autophagy inhibition differed depending on whether Trp53 was concurrently deleted or not (19). Some of these differences could be due to the models used, or, alternatively, that particular ATG genes may have nonoverlapping
functions that are independent of autophagy. In addition, there are likely differences regarding whether \( \text{Trp53} \) is deleted homozygously in the germline, or if one copy is lost by somatic LOH (mimicking the cognate human phenomenon). Our data are consistent with prior reports showing that loss of autophagy can promote the initiation of tumorigenesis in the pancreas, and prevent the progression to cancer in the setting of oncogenic \( \text{Kras} \) mutations (19). However, unlike the data from Rosenfeldt and colleagues (19), our work shows that \( \text{Atg5} \) deletion impairs the progression of premalignant PanIN to invasive PDAC in the setting of \( \text{Trp53} \) loss. We believe that this difference stems from the fact that in their study, \( \text{Trp53} \) was homozygously deleted during embryogenesis. Therefore, in the physiologic setting of \( \text{Trp53} \) loss during
tumor progression, autophagy seems to be required for optimal PDAC development. The intricate and complex relationship of autophagy and p53 is of great importance and awaits further study (26).

Perhaps most relevant to cancer treatment, we showed that acute inhibition of autophagy by CQ treatment or RNAi inhibited growth of murine PDAC cell lines with various Trp53 alterations, and is consistent with our prior data using human PDAC cell lines, which almost all harbor TP53 mutations (2). Finally, we used a large panel of patient-derived PDAC xenografts and performed treatment studies using HCQ. HCQ treatment attenuated the growth of the majority of primary patient-derived PDAC xenografts that harbor TP53 mutations.

Together, our data continue to support the integration of autophagy therapies into the treatment of PDAC. Ongoing human clinical trials in PDAC will determine whether this approach is feasible and effective in patients.

METHODS
Genetically Engineered Mice
Atg5−/− mice were kindly provided by Dr. Noboru Mizushima (The University of Tokyo, Tokyo, Japan; ref. 27). Trp53−/− mice were obtained from Anton Berns (Netherlands Cancer Institute, Amsterdam, The Netherlands; ref. 28). Pdx1-Cre was obtained from Doug Melton (Harvard University, Boston, MA; ref. 29). All animal experiments were approved by the Institutional Animal Care and Use Committee under protocol 10-055 at the Dana-Farber Cancer Institute (Boston, MA). Mice were maintained on a mixed background. Survival was determined by humane endpoints as specified by the protocol, including showing signs of being moribund, significant weight loss, skin ulceration, or in rare cases being found dead. All mice with PDAC-specific death were histologically confirmed.

Histology
All tissues were fixed in 10% formalin overnight and embedded in paraffin. For IHC, tumors were deparaffinized and rehydrated. After antigen retrieval in citrate buffer (pH, 6.0), tumors were labeled with primary antibody overnight and then detected using the VECTASTAIN Elite ABC Kit (pk-6100; Vector Labs) and DAB (sk-4100; Vector Labs). Antibodies used for immunohistology are labeled with primary antibody overnight and then detected using secondary antibody for 1 hour and exposed on film using the Enhanced Chemiluminescence (ECL) Detection System (Thermo Scientific). Antibodies used were as follows: ATG5 (1:500; NB110-53818; Novus Biologicals), ATG7 (1:500; A2856; Sigma), LC3 (1:500; NB600-1384; Novus Biologicals), p53 (1:1,000; FL-393; Santa Cruz Biotechnology), and β-actin (1:3,000; A0266; Sigma).

Lentivirus-Mediated shRNAs
All shRNA vectors were obtained from the RNA Interference Screening Facility of Dana-Farber Cancer Institute. Atg5 (TRCN0000057819) and Atg7 (TRCN0000092163) shGFP; forward 5′, CCGGCGCAAGCTGACCCTGAGTTCATTCAAGAGATGAACTTCA GGTCAAGCTTGGGCCAGCTAG (TRCN0000092163); backward 3′, AATTAAAAAGCAAGCTGGAC CTCGAAATTCTTCTTTAGATGAATGAGT (TRCN0000057819). Lentivirus was produced using 293T cells, as previously described (2).

Electron Microscopy
Freshly harvested subcutaneous tumors from mice were fixed immediately with 3% paraformaldehyde, 1.5% glutaraldehyde, and 2.5% sucrose in 0.1 mol/L sodium cacodylate and cut into 1-mm³ squares. After postfixation in 1% osmium tetroxide for 1 hour on ice and dehydration, the samples were embedded in a mixture of Epon–araldite. Thin sections from four blocks were collected on uncoated grids, stained with uranyl and lead citrate. Samples were sectioned and examined using an FEI Tecnai 12 Transmission Electron Microscope equipped with a 16-bit 2K × 2K FEI Eagle bottom-mount camera and an ISIS MegaView III wide-angle camera. Images were captured at ×12,000 magnification (32).

Clonogenic Assay
Cells were plated in 6-cm dishes at 500 cells per dish in growth medium with 10% FBS and treated with CQ the day after seeding. After 7 days, cells were fixed in 80% methanol and stained with 0.2% crystal violet, and colonies were counted. The surviving fraction was calculated using the plating efficiency.

Growth Curves
Cells were plated in 24-well plates at 3,000 cells per well in 1 mL of media. Media were not changed throughout the course of the experiment. At the indicated time points, cells were fixed in 10% formalin and stained with 0.1% crystal violet. Dye was extracted with 10% acetic acid, and the relative proliferation was determined by attenuation (D) at 595 nm.

Oxygen Consumption Measurements
Oxygen consumption measurements: 1.5 × 10⁶cells were seeded in a 96-well Seahorse plate, and OCRs were measured using the Seahorse XF96 instrument (Seahorse Biosciences). Basal mitochondrial respiration (3 mmol/L glucose) and ATP production (2 mmol/L oligomycin) were measured. Maximal respiration was obtained by adding carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP; 0.5 mmol/L), and non-mitochondrial OCRs were obtained by adding 2 mmol/L antimycin A. Values were normalized by protein concentration to account for cell number.

Western Blot Analysis
Proteins were extracted by RIPA buffer and separated on 4% to 12% stacking SDS-PAGE gel. Proteins were then transferred to polyvinylidene difluoride (PVDF) membrane (Bio-Rad). Membranes were blocked with 5% nonfat dry milk and then incubated with the primary antibody overnight at 4°C. Following Tris Buffered Saline with Tween 20 (TBST) washing, membranes were incubated with peroxidase-conjugated secondary antibody for 1 hour and exposed on film using the Enhanced Chemiluminescence (ECL) Detection System (Thermo Scientific).
In Vivo Efficacy of HCQ in Human PDXs

Animal experiments were conducted following approval and in accordance with the Institutional Animal Care and Use Committee guidelines of the Johns Hopkins University under protocol M006M385. A total of 12 human PDXs established from the primary tumors, resected from patients with pancreatic cancer at the Johns Hopkins Hospital, were used for the study (33). The mutational status of these tumors was previously reported (14) and is shown as Supplementary Table S1. Fresh tumors resected from mice were cut into cubes of 2 mm³, and were subcutaneously implanted on both flanks of 6-week-old female nu/nu athymic mice (Harlan). When cohorts of tumors reached approximately 150 mm³, animals were randomly assigned to receive vehicle or HCQ (60 mg/kg, i.p., once daily for 4 weeks) treatments (2). Tumors were measured twice per week, and tumor volumes were calculated using the following formula: \( V = \frac{a \times b^2}{2} \), where \( a \) is the largest dimension and \( b \) the smallest. Tumor growth in HCQ-treated animals was compared with that in vehicle-treated mice.

Statistical Analysis

Overall survival events included death as defined by the protocol with censoring for alive at last follow-up. Events for PDAC-specific survival included deaths attributable to PDAC with uninformative censoring for deaths related to other causes or at last follow-up. Survival plots were generated using the Kaplan–Meier method. The log-rank test was used to compare survival distributions between groups. The proportion of mice alive at 30 weeks or longer in the AgS5/L/L group was compared with the other two groups using a Fisher exact test. Statistical analyses were performed using R version 3.0.2 (34).

Disclosure of Potential Conflicts of Interest

A.C. Kimmelman has received honoraria from the speakers’ bureau of US Oncology and is a consultant/advisory board member for Forma Therapeutics and Gilead. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions

Conception and design: A. Yang, N.V. Rajeshkumar, B.M. Alexander, A. Maitra, A.C. Kimmelman

Development of methodology: A. Yang, N.V. Rajeshkumar, B.M. Alexander, A. Maitra

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): A. Yang, S. Yabuuchi, A. Maitra

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