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Cancer Research

The Damaging Effect of Passenger Mutations on Cancer Progression

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Abstract

Genomic instability and high mutation rates cause cancer to acquire numerous mutations and chromosomal alterations during its somatic evolution; most are termed passengers because they do not confer cancer phenotypes. Evolutionary simulations and cancer genomic studies suggest that mildly deleterious passengers accumulate and can collectively slow cancer progression. Clinical data also suggest an association between passenger load and response to therapeutics, yet no causal link between the effects of passengers and cancer progression has been established. To assess this, we introduced increasing passenger loads into human cell lines and immunocompromised mouse models. We found that passengers dramatically reduced proliferative fitness (~3% per Mb), slowed tumor growth, and reduced metastatic progression. We developed new genomic measures of damaging passenger load that can accurately predict the fitness costs of passengers in cell lines and in human breast cancers. We conclude that genomic instability and an elevated load of DNA alterations in cancer is a double-edged sword: it accelerates the accumulation of adaptive drivers, but incurs a harmful passenger load that can outweigh driver benefit. The effects of passenger alterations on cancer fitness were unrelated to enhanced immunity, as our tests were performed either in cell culture or in immunocompromised animals. Our findings refute traditional paradigms of passengers as neutral events, suggesting that passenger load reduces the fitness of cancer cells and slows or prevents progression of both primary and metastatic disease. The antitumor effects of chemotherapies can in part be due to the induction of genomic instability and increased passenger load. *Cancer Res; 77(18); 4763–72. ©2017 AACR.*

Introduction

Genomic instability, that is, a high frequency of mutations and chromosomal alterations (referred to collectively as mutations) within cellular lineages, is a hallmark of carcinogenesis (1). Genomic instability creates both driver and passenger mutations. Drivers are defined as mutations that confer a fitness advantage to somatic cells in their microenvironment, thereby driving the cell lineage to cancer (2). Conversely, passengers (also termed "hitchhikers") are defined as mutations that provide no such proliferative benefit (2). Recurrence patterns in sequenced cancers estimate that the vast majority (97%) of mutations in cancer are passengers (3). Compared with drivers, however, little is known about passengers. Accordingly, recent literature disagrees over the properties of classified passengers, with some arguing that passengers are misclassified "mini-drivers" (4), or latent drivers (5), or effectively neutral (6, 7), and potentially deleterious to cancer (8, 9).

These diverging hypotheses for passenger's effect, although nonexclusive, result from a few surprises and limitations in whole-genome analyses. First, the number of newly identified driver genes was less than expected (4). Second, some sequenced cancers genomes have very few or no known drivers (10). Finally, accumulation-based statistics (e.g., dN/dS) cannot distinguish "effectively neutral" mutations, a term initially applied to sexually evolving populations (11), from "mildly deleterious" mutations in asexuals like cancer ("mildly deleterious" mutations are operationally defined by accumulation statistics; ref. 12). All of these findings underscore the need to directly measure passenger's phenotype.

Our recent analyses of human cancer genomics data (8, 9) indicate that accumulated passengers, an inextricable consequence of genomic instability, can be moderately deleterious to cancer cells. These deleterious passengers largely evade natural selection in our evolutionary models (Fig. 1). Our model considers individual cells with the capacity to acquire advantageous drivers and deleterious or neutral passengers. Mildly deleterious passengers, alongside rare but strongly advantageous drivers, proved to be most consistent with genomic and epidemiologic data (9). Although passengers exhibit individually weak effects on progression, their cumulative effect can commensurate with that of drivers because of their disproportionately high numbers,

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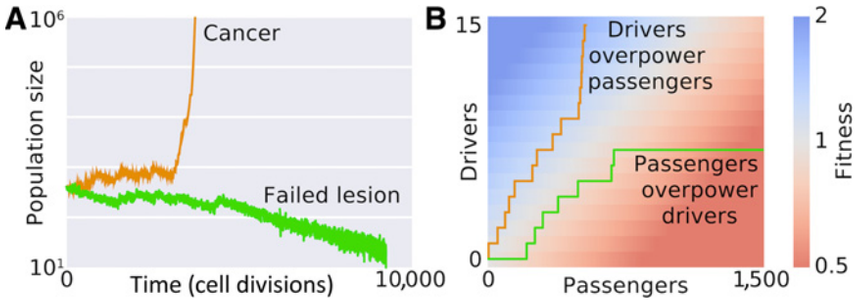

Figure 1.

Accumulation of passenger mutations can slow cancer progression and lead to cancer meltdown. **A,** Time course of cancer development from the deleterious passenger model (4, 5). In the model, cancer cells can acquire both strong advantageous drivers and mildly deleterious passenger mutations. Cells divide and die stochastically depending on their fitness, while other aspects of phenotype are not explicitly modeled (advantageous phenotypes, i.e., drivers that increase cell division are mathematically equivalent to phenotypes that avoid cell death or expand a tumor's microenvironment in our formalism; ref. 8). Because of a tug-of-war between drivers and passengers, initially identical lessons can either progress to cancer or regress to extinction. **B,** Tumor fitness and population size in this model is determined by the relative abundances of drivers and passengers. Deleterious passengers accumulate in all populations despite negative selective pressures. Successful tumors acquired drivers disproportionately faster than passengers.

leading to a tug-of-war between drives and passengers (8, 9). As such, passengers can reverse and prevent tumor growth in models where population size can freely fluctuate (Fig. 1). Passenger's deleterious effects are most pronounced at elevated mutations rates, a phenomenon termed mutational meltdown in other evolving asexual populations (13). The predictions of these evolutionary models, however, remain experimentally untested.

Other recent studies find that passengers can increase tumor immunogenicity (14–17) and that very high genomic instability correlates with improved clinical outcomes (14, 18, 19, 20) or reduced cell proliferation (21, 22). However, most of these studies focus on single-nucleotide variants or whole chromosomal gains and losses, and provide a correlative, but not causal, link between mutational load and clinical response.

Here, we developed human cell line and mouse models to directly assess the effects of passenger load on cell fitness and carcinogenesis. We found that passengers were deleterious to cell fitness and tumor progression in a manner independent of the immune system. We identified a metric of the aggregate passenger load of chromosomal alterations that best explains the effects of passengers on the fitness of cell lines and human cancers. In mouse models, we demonstrated that genomic instability considerably slows tumor growth and that elevated passenger load reduces metastatic burden. Our findings indicate that the cumulative effect of passenger events is damaging to cancer cells, affecting cancer progression, and suggest potential therapeutic avenues to exploit deleterious passengers.

Materials and Methods

In vitro fitness assay

MCF10A cells were obtained from the ATCC. NeuT was transmitted via retrovirus and control empty virus (under blasticidin selection) as in our prior publications (23) and Her2 expression was confirmed (data not shown). Two days postinfection, mild blasticidin (10 μg/mL) selection was used to ensure transformation. Growth rates, that is, inverse doubling time, were measured by direct cell counting on a glass plate for 2 days. DNA, for genotyping, was isolated and prepared as in our previous publication (24).

Metastatic assay of MCF-10A cells with increasing passenger load

MCF-10A cells preinjection were treated with doxorubicin as described above. However, in this experiment, there was no cloning after mutagenesis; instead, cells were given a 6-day drug-free recovery period. Lower growth rates for both treatment groups (10 nmol/L – 20.38 hours and 20 nmol/L – 20.81 hours) relative to untreated control cells (18.90 hours), postrecovery were confirmed, as seen in the first experiment. Cells (2.5×10^6), with stable expression of firefly luciferase, were injected into the tail vein of female SCID mice and imaged once per week for 7 weeks. Thoracic-region bioluminescent signal was determined (photons/second ± SEM). *Ex vivo* bioluminescence of surgically resected tumors was imaged and individual metastases, defined as isolated bioluminescent foci, counted. *Ex vivo* bioluminescence agreed well with *in situ* thoracic-region bioluminescence at week 7.

Mouse model of tumor progression with elevated mutation rates

Experiments were performed using an MMTV-neu mouse model of Her2-positive breast cancer.

MMTV-neu (F) mice were crossed with homozygous $H2AX^{-/-}$ (M) mice to generate $neuT^{+/-}H2AX^{+/-}$ haploinsufficient progeny defective in repair of single- and double-strand DNA breaks (24). MMTV-neu (F) mice were also crossed with control FVB/NJ (M) mice to generate $neuT^{+/-}H2AX^{+/+}$ progeny. Tumor emergence and sizes were measured every other day. After tumors grew to appreciable size, animals were sacrificed and tumors were removed. DNA was prepared as described above.

All animal care, experimentation, and sacrifice were conducted humanely in compliance with ethical standards.

Human and mouse genotyping

Genotyping was performed using a combination of Affymetrix Genome-wide Human SNP Arrays 6.0 and low-coverage (mean 0.4×) DNA sequencing (Illumina HiSeq 2000). This depth permitted CNV calls at a resolution of 20 kb. Reads were mapped to HG19 and MM9 (see Supplementary Information). Metastatic samples were not genotype because, after the first experiment, we found that growth rate correlated more closely with doxorubicin exposure ($r = -0.89$) than our best genomic-based metric of passenger load (LASSO model, $r = 0.87$).

DNA copy number was determined using the GLAD software package (for SNP arrays) and cn.MOPS (for low-coverage sequencing; Supplementary Tables S1 and S2; ref. 25). Copy number alterations (CNA) were then identified by integer transformation of the copy number tracks (assuming no stromal contamination, nor subclonal alterations; see Supplementary Information). All samples were near-diploid. CNAs were called using two approaches: a high-specificity set and a high-sensitivity set. The latter proved more internally reproducible and also more consistent with mutagen exposure (Supplementary Fig. S1).

Ancestral CNAs (existing in the cell lines prior to experimentation) were removed from MCF-10A lines by identifying an ancestral genome using a maximum parsimony approach (see Supplementary Information). We did not expect and did not find any shared CNAs between mouse samples, as the mice had stable diploid genomes.

Genomic analysis

Putative drivers were identified using recurrence data from prior large-scale human breast sequencing projects [The Cancer Genome Atlas (TCGA)]. A list of putative oncogenes and tumor suppressors (Supplementary Table S3) was identified using the GISTICII algorithm (3) thresholded to $P < 0.001$. For mouse analyses, the identified human drivers were mapped to their close mouse homologs via BioMart (Supplementary Table S3; all genomic annotations in humans had good homology to mice; ref. 26). For an alteration to be annotated as a driver, it was required to either be an amplification that (at least partially) overlapped an oncogene, or be a deletion that (partially) overlapped a tumor suppressor. Driver events, by design, were rare in our experimental cell lines (Supplementary Tables S1 and S2) and did not appreciably alter passenger load or cell-doubling time.

We explored a wide variety of genomic features that were perturbed by passenger alterations and how these perturbations might, quantitatively, affect cell fitness. A total of 3,804 human housekeeping genes were identified in a previous study (27) and used alongside annotations of all open reading frames (ORF; ref. 28) to develop measures of housekeeping disturbance and total gene disturbance, that is, the number of (housekeeping) genes altered by a passenger weighted by their copy number change. Copy number change is the absolute difference between the observed ploidy and 2 (euploid/diploid). Gene expression levels (average of replicates) of Her2-activated and untransformed MCF-10a cell lines were identified previously (29) and used for estimating passenger load. Expression disturbance was calculated by weighting the expression level of every ORF altered by a passenger (sum of expression of each ORF) weighted by the copy number change of the passenger. Focal CNAs were defined as CNAs that neither began nor ended in a telomere-annotated region or a centromere-annotated region, as defined by the Human Genome Browser at University of California, Santa Cruz (Santa Cruz, CA; ref. 28). Finally, 95 genotyped and sequenced human breast cancers were also studied and obtained previously (30).

A LASSO-regularized linear model was constructed from a combination of all of the above features to predict cell-doubling time (for MCF-10a cell lines) and to predict driver load (for sequenced TCGA breast cancers). Regression fits were calculated numerically via the scikit-learn package (31), and a consensus fit of 10,000 randomly initiated iterations was obtained for every model. An L1 weight of $\alpha = 10^{-3}$ was chosen as this value preceded the downward inflection in adjusted R^2, relative to increasing α, that is typically seen during model selection (Supplementary Fig. S3; ref. 31).

Simulations

A first-order Gillespie algorithm (8) modeled individual cells in a precancerous population. Cells could divide and die at rates determined by population size and their internal genotypes, which acquired advantageous driver and deleterious passengers during division. Phenotypes other than cell fitness were not explicitly modeled. Driver phenotypes that increase cell division, avoid cell death, or expand the tumor microenvironment (e.g., via invasion) are mathematically equivalent in our formalism.

Results

Effect of passengers on proliferative fitness of cancer cells

To directly test for the effects of passenger load on proliferative fitness in cancer cells, we developed cell lines with nearly identical drivers and controlled passenger loads (Fig. 2A). First, spontaneously-immortalized, and genomically stable human breast epithelial cells (MCF-10A) were transformed with a single driver, activated Her2/Erbb2 oncogene, using a retroviral expression system (24, 32). To ensure genomic homogeneity of the initial population, an individual clone was isolated after transformation, and alterations in this clone were inferred and used as a baseline for subsequent genomic analysis (Supplementary Fig. S1). Cells from this clone were treated with different doses of doxorubicin at sublethal, subclinical levels (0–30 nmol/L) overnight. The time of exposure to mutagen was less than the time needed for a single cell division; therefore, selective forces in the mutagenic environment should be minimal. Doxorubicin, a topoisomerase II inhibitor, introduces CNAs, mimicking natural genomic instability (33), and at rates several orders of magnitude higher than point mutations (34). Activation of the Her2 pathway suppresses repair of these alterations by suppression of double-strand break repair (24). Cell lines were then given a 2-week recovery period to eliminate any residual doxorubicin toxicity and cellular stress. Individual clones were isolated from each mutagenized population to ensure genomic homogeneity within each sample (Fig. 2A). As a control, untransformed MCF-10A cells were subjected to the same protocol.

In agreement with our hypothesis, increasing passenger load negatively correlates with doubling time; cells with the highest passenger load (20 and 30 nmol/L doxorubicin exposure) grew >30% slower than unmutagenized strains (Fig. 2B). Doubling times were measured by daily cell counting on plates for 3 days. Mutagen exposure alone cannot explain this growth reduction as untransformed MCF-10A cells (with functioning DNA repair) exposed to doxorubicin accumulated few additional passengers and did not show any reduction in growth rate (Fig. 2C). Taken together, these results show that the proliferative fitness of cancer cells declines with accumulated passenger load.

Genotyping of the developed cell lines, using a combination of SNP array and low-coverage whole-genome sequencing, confirmed that (i) increasing doxorubicin levels incur greater quantities of alterations that are classified as passengers by existing methods, that (ii) very few additional known drivers accumulated (mean 1.4/sample), and that (iii) accumulated passengers did not avoid functional genomic elements, such as housekeeping genes, or genes in general. Similarly, clinical passengers do not avoid these functional elements (Supplementary Table S1; Fig. 3A).

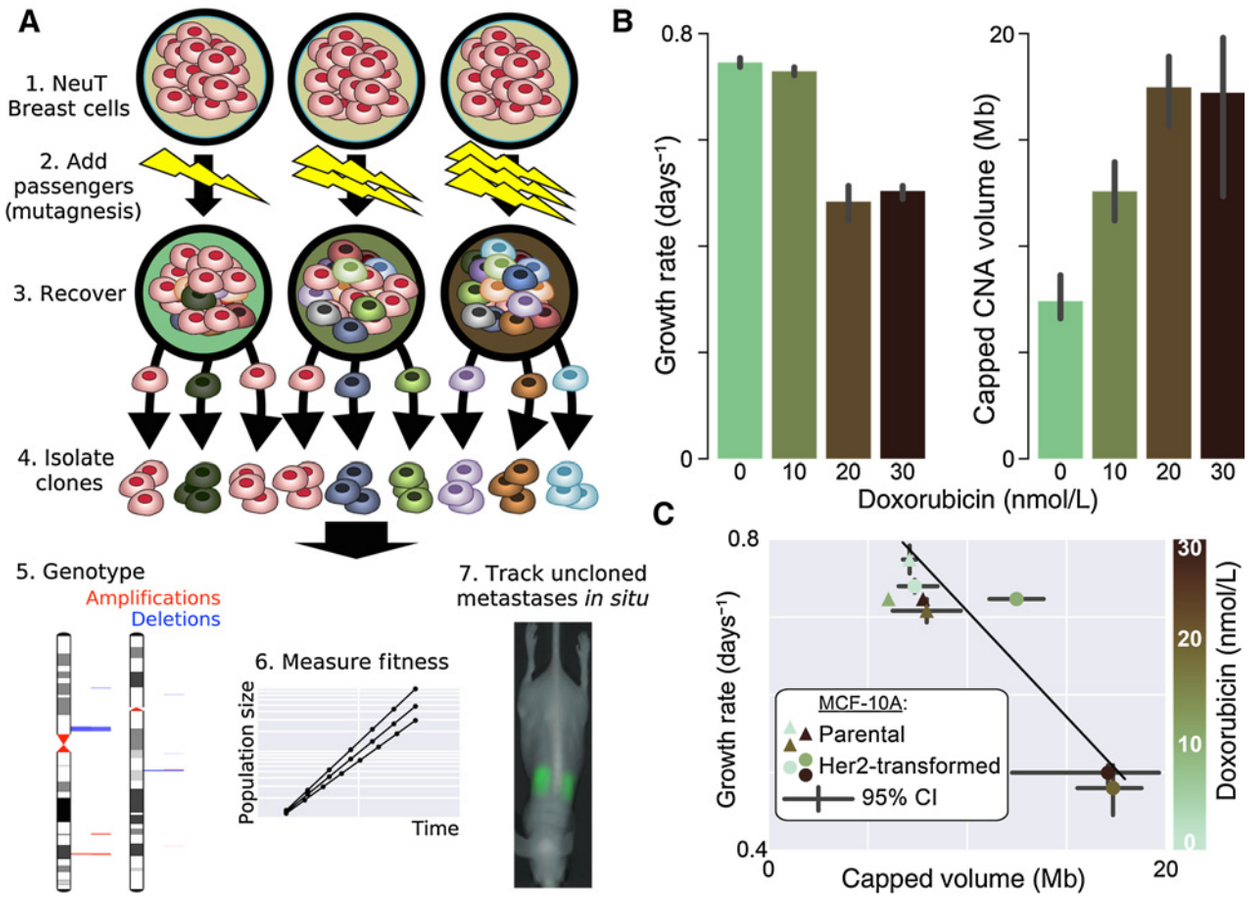

Figure 2.

Passenger alterations reduce proliferative fitness of cancer cells. **A,** Experimental design to produce cell lines with identical drivers and increasing loads of passengers. Passenger alterations were introduced into Her2-transformed MCF-10A breast cells by low-dosage mutagenic doxorubicin. After recovering for 2 weeks, clones were isolated, genotyped for CNAs, and assayed for cell fitness and metastatic potential (Fig. 4). **B,** Increasing dosages of mutagen decrease proliferative potential and increase passenger load, which was summarized by capped CNA volume (Materials and Methods; Supplementary Figs. S2 and S3). **C,** Fitness effects of cells lines versus passenger load. Data points represent the average fitness and passenger load of biological replicates at various concentrations of doxorubicin exposure. The fitness cost of passengers in transformed cells was 0.028 per Mb ($r^2 = 84\%$; 95% CI, 64–99). Untransformed cells neither acquired passengers nor decreased in proliferative potential, suggesting that passengers, not doxorubicin toxicity, reduce fitness. Error bars (95% CI) and *P* values were calculated using bias-corrected bootstrapping throughout this study (50).

Although mutations generally do not accumulate in these regions in natural populations, the deleterious passenger model argues that the selective forces that weed-out these mutations are suppressed in cancer (8). Her2-transformed clones treated with 20 to 30 nmol/L doxorubicin acquired on average 296 novel alterations that would be classified as passengers by existing algorithms, significantly higher than the untransformed MCF-10A cells exposed to same doxorubicin levels ($P < 0.005$; Supplementary Table S1). Thus, these lines constitute an ideal system to study the effects of increasing passenger loads on cell with nearly identical drivers (Materials and Methods; Supplementary Table S2).

Evaluating fitness effect of accumulated passengers

Next, we asked whether passenger load could quantitatively predict reduction in proliferative fitness of cancer cells. We considered several measures of passenger load (detailed below) that summarize various properties of these alterations and their effects (Fig 3B). Every measure positively correlated with cell-doubling time, strongly supporting the notion that passenger load slows tumor growth (Fig. 3C).

Measures, broadly speaking, can consider properties of the CNAs themselves, their length, copy number, and termini, or they can consider properties of the genes altered by each CNA. Gene-naïve measures can simply consider the number of CNAs, or they can weight each CNA by its length and absolute change in copy number, termed CNA volume (Fig. 3B; refs. 35, 36). Because CNAs vary tremendously in length (Supplementary Fig. S3A), we also considered volumetric measures of CNAs that attenuate the weight given to very long CNAs, for example, capped CNA volume (which caps the weight of a CNA at 2 Mbs).

Of the gene-naïve measures, measures that weight CNAs by their length, but attenuate weights for very long CNAs, predict fitness effects best (Fig. 2C). Presumably, longer CNAs disturb more genetic elements and, thus, incur greater fitness cost.

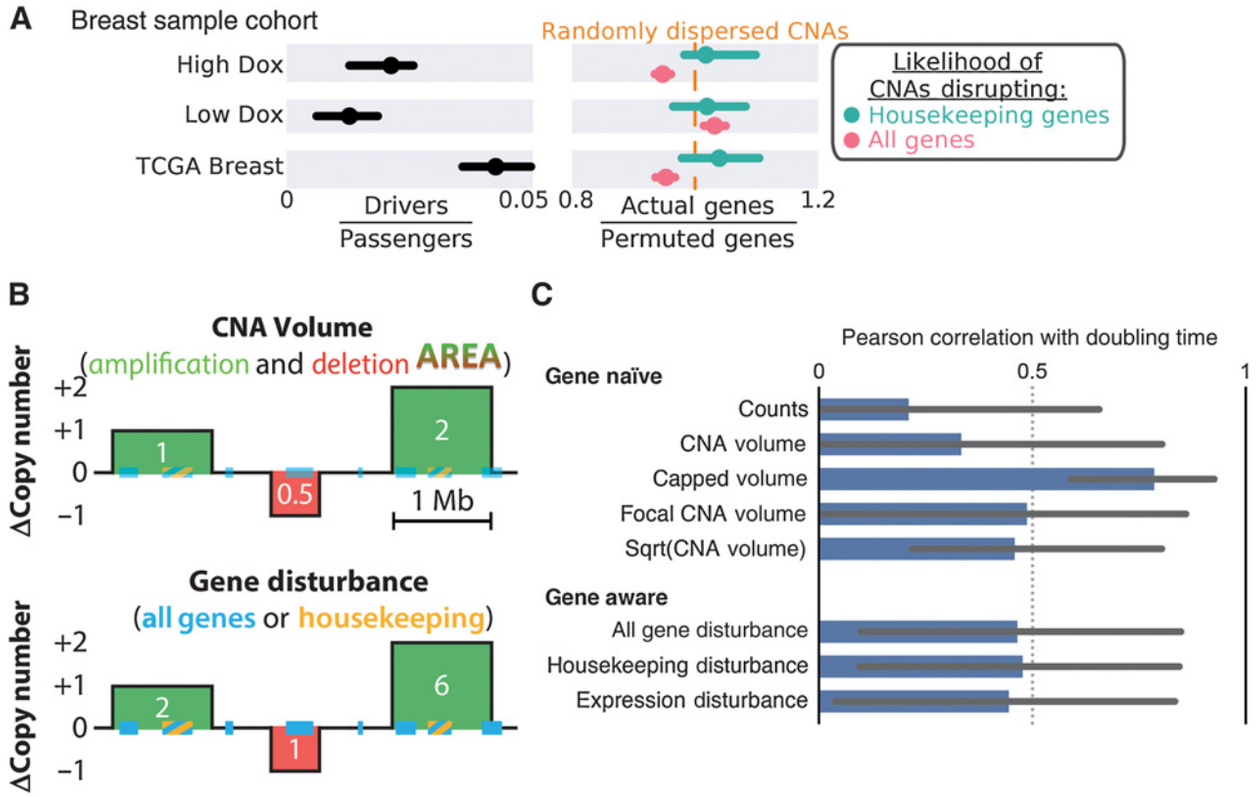

Figure 3.

Measures of passenger load predict experimental cell line fitness and mutational patterns in TCGA breast cancers. **A,** Left, passenger alterations dominated the genomes of experimental cell lines, as intended [low doxorubicin (Dox), 0/10 nmol/L; high doxorubicin, 20/30 nmol/L doxorubicin]; right, passenger alterations in both our cell lines and TCGA cancers span genes (and housekeeping genes in particular) at rates approximately expected by random chance (determined by randomly permuting gene locations across all regions not annotated as centromeres or telomeres; ref. 28). Housekeeping genes were identified previously based on ubiquitous expression (27). **B,** Passenger load, the fitness cost of all passengers in a genome, may depend upon the properties of individual passengers. CNA volume weights each alteration by its deviation from normal copy number and its length. Gene (housekeeping) disturbance weights each alteration by its deviation from normal copy number and the number of (housekeeping) genes perturbed. **C,** All passenger load measures negatively correlated with cell line growth rate. Capped CNA volume was most predictive.

However, these fitness costs attenuate for very long CNAs in our cell line experiments and analyses of clinical breast cancers (see below). Although capped CNA volume proved to be the most predictive, all measures negatively correlated with growth rate ($P < 0.05$; Supplementary Fig. S2C), indicating that passengers are deleterious by any measure.

The attenuation of fitness costs for very long CNAs was unexpected, so we considered two possible explanations. First, we suspected that nonfocal CNAs, alterations that either begin or end at a centromere or telomere, might confer weaker fitness effects per Mb than focal CNAs, as they are categorically different lengths (Supplementary Fig. S2A) and less likely to form gene fusions. Ignoring nonfocal CNAs predicted cell fitness better than raw CNA volume, but still worse than attenuated CNA volume metrics, supporting this hypothesis. Alternatively, very long CNAs may only accumulate in the tumor population when their fitness cost is considerable. If so, then the very long CNAs that do accumulate should be less deleterious. Indeed, very long CNAs in clinical cancer samples appear to be depleted relative to a null model of CNA mutations (35). Therefore, the location of CNA termini and selection against very long CNAs may explain the attenuated fitness costs of very long CNAs.

We then tested "gene aware" metrics of passenger load. We considered (i) the number of altered genes, termed gene disturbance; (ii) the number of altered housekeeping genes, termed housekeeping disturbance, and (iii) the number of altered genes weighted by their expression termed expression disturbance. For all of these measures, each passenger's effect was also weighted by the absolute change in copy number of affected genes (see Materials and Methods). All of these measures predicted fitness of cells with variable passenger load better than CNA volume, but not appreciably (Fig. 3C). This suggests that (essential) genes and their altered expression partially explain passenger load, but that the passenger's harm extends beyond coding regions of the genome.

We further validated these characteristics of passenger load using clinical breast cancer data. Our evolutionary tug-of-war model predicted (9) that passenger load must be counterbalanced by additional drivers (Supplementary Information). Consistent with these predictions, we observed a positive linear relationship

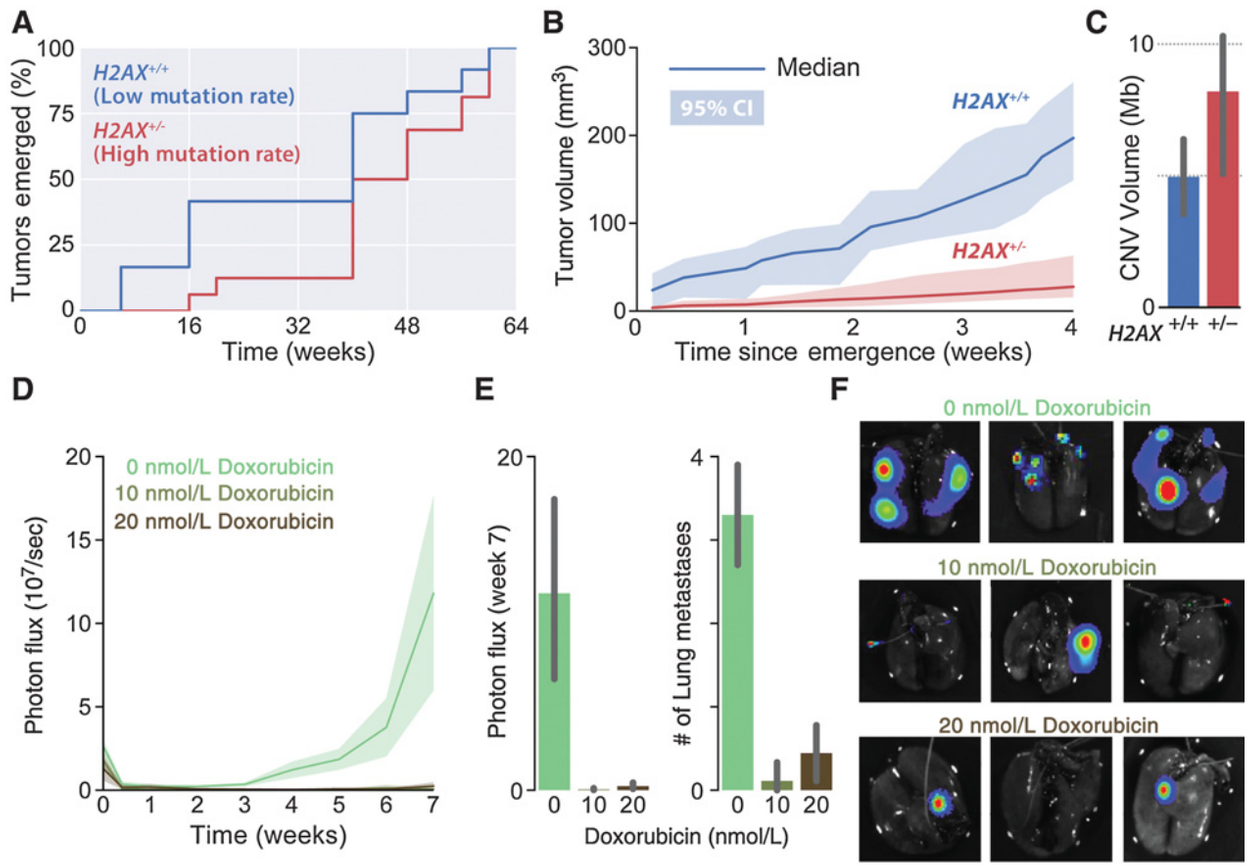

Figure 4.

Elevated passenger load slows tumor growth and reduces metastasis in mice. **A,** Her2-positive breast cancers emerged from mice in the highly mutagenic ($H2AX^{+/-}$) line at similar times as in the moderately mutagenic ($H2AX^{+/+}$) line (possibly later, $P = 0.05$). **B,** Tumors with elevated genomic instability (high mutation rate) grew 52% slower (95% CI, 40–66) after emergence. Shaded regions, 95% CI of growth determined via bootstrapping. **C,** Genotyping confirmed that elevated mutation rates increase passenger loads. **D,** Growth of lung metastases created by injecting Her2-transformed MCF-10A breast cells with differential passenger loads (described previously). Cells were transfected with a luciferase reporter before mutagenesis to enable *in situ* thoracic bioluminescence monitoring and then injected into the tail vein of SCID mice (10 per condition) without cloning. **E,** At 7 weeks, total metastatic burden was measured and lung metastases were counted by dissection. **F,** Representative *ex vivo* bioluminescent lung images.

between simple passenger load (e.g., number of nonsynonymous passenger mutations) and the number of identified drivers in cancer genomics data (9). Our improved measures of passenger load developed here can also be evaluated on their ability to correlate with the number of driver mutations in cancer genomics data. We found that our measures of passenger load, and capped CNA volume in particular, indeed exhibited improved linear relationships with the number of driver events (Table 1), further supporting the tug-of-war between drivers and passengers (8, 9).

Finally, we developed a LASSO-regularized, combined linear model that compared all passenger load metrics, including gene-naïve and gene-aware measures, to predict cell fitness (doubling time) and the driver load (Table 1; Supplementary Table S3). Our combined model outperformed any single metric (even after correcting for degrees of freedom) when predicting either cell-doubling time in our experimental cell lines or driver load in clinical breast cancers (adjusted $R^2 = 0.63$ and 0.74, respectively). Thus, carefully combining measures improves predictions of cell line fitness and driver load better than any single metric. In this combined model, gene-aware measures were most useful, suggesting that the most accurate description of passenger fitness cost must consider multiple genomic properties of each passenger.

By vetting passenger load metrics on two orthogonal datasets with two approaches, we strengthened the conclusions of our fitness predictions. Passenger metrics that predicted cell-doubling time better also predicted driver load better (Spearman correlation = 0.6, $P < 0.001$) and were also more informative to combined linear models. This consilience, which cannot be due to overfitting of a particular dataset, favors capped CNA as the most useful single metric.

Developed cell lines that carry differential passenger loads and the same drivers also allow us to directly measure the fitness cost of passenger alterations. By regressing the growth rate to capped CNA volume, we find a mean fitness cost of 0.027 per Mb [95% confidence interval (CI), 0.0213–0.056; $P < 0.0001$; Fig. 2C], that is, an approximately 2.7% per Mb growth reduction for <2 Mb CNAs and an approximately 5% growth reduction per CNA longer than 2 Mb. This measurement is in excellent agreement with our

Table 1. Performance of passenger load metrics

	Pearson correlation (%)		Weight in LASSO-regularized, linear model (%)	
Passenger load metric	**Doubling time[a]**	**Driver load[b]**	**Doubling time[a]**	**Driver load[b]**
Gene naïve				
Counts	21	42	<1	1
CNA volume	33	54	0	4
Volume (2 Mb cap)	78	69	9	25
Focal CNA volume	48	54	0	2
Log (CNA volume)	21	67	0	0
Sqrt (CNA volume)	46	66	8	<1
Gene aware				
All gene disturbance	46	61	61	36
Housekeeping disturbance	47	60	8	31
Expression disturbance	44	59	14	0

NOTE: Passenger load metrics predict cell line fitness and TCGA mutational patterns in isolation and in a combined model. The deleterious passenger model predicts that the tug-of-war between drivers and passengers imparts a positive linear relationship between drivers and passenger load on cancer genomes (Supplementary Information; ref. 9). All measures identify this relationship in TCGA breast cancers. Combining measures into a LASSO-regularized linear model improves predictions of cell line fitness and driver load better than any single metric (adjusted $R^2 = 0.63$ and 0.47, respectively).

Abbreviation: Sqrt, square root.

[a]Experimental cell line data.

[b]TCGA breast samples.

earlier inference of fitness loss per Mb for CNA in human cancers (2%–10% depending on the chromosome), which was based on the analysis of approximately 40,000 intrachromosomal arm CNAs from more than 3,000 cancer specimens and on chromosome conformation capture, Hi-C, data (35).

Moreover, this estimated CNA fitness cost further allows us to quantify the total passenger load of human breast cancers. We calculate that on average, a breast cancer sample has a capped CNA volume of 146 Mb that translates into an estimated >300% hindrance of tumor growth by passenger CNAs. This dramatic effect of passengers helps to explain some observations in the literature, in particular, why tumors with high levels of alterations have better prognosis than those with moderate levels (18, 37) and, most recently, that this is a general phenomenon across cancers (20).

Although seemingly high, this large collective effect of passengers is consistent with previous evolutionary modeling and genomic analyses, which find that drivers and passengers are in a delicate balance and the total fitness cost of passengers is barely outweighed by the collective benefit of drivers (each driver leading to a 20%–60% average increase of fitness; refs. 9, 38). Taken together, results of the cell line experiment combined with genomic analysis show that cancers carry a high passenger load that substantially reduces cell fitness.

Effect of passengers on tumor growth in mice

To investigate the effects of passengers on other aspects of cancer development, emphasizing clinical utility, we turned to mouse cancer models. We first used transgenic mice to investigate the effects of increased genomic instability [inducible by cytotoxic (39) and targeted (40) chemotherapies] on tumor growth. Unlike traditional paradigms, where genomic instability always accelerates carcinogenesis (1, 41), we predicted that tumor growth can be slowed or even suppressed when mutation rates exceed a critical level (Supplementary Information; ref. 9). To test this prediction, we created mouse models of breast carcinogenesis with high and low alteration rates by crossing an MMTV-neu mouse model of Her2-positive breast cancer [mice containing a single driver, activated Her2 (NeuT) expressed in the mammary epithelium; ref. 24], with mice containing a homozygous deletion of histone H2AX that is necessary for DNA double-strand break repair (Supplementary Information; ref. 32). Hybrid progeny carried a single copy of the NeuT oncogene and were H2AX haploinsufficient. As a control, we used animals that also carried a single copy of NeuT and both copies of *H2AX* gene. Tumors emerged after a median of 10 months in both $H2AX^{+/-}$ and control mice (Fig. 4A).

Strikingly, we found that cancers grew significantly slower ($P < 0.001$) in mice with high mutation rates relative to control tumors (Fig. 4A and B), thereby demonstrating that (i) passengers are deleterious in organismal environments, and that (ii) genomic instability can be detrimental to tumor growth (1, 42). Low-coverage sequencing of seven isolated, uncloned tumors identified a nonsignificant increase in accumulated passengers in $H2AX^{+/-}$ mice, as expected (Fig. 4C, $P = 0.11$, two-sided t statistic of capped volume). One $H2AX^{+/+}$ tumor appeared to acquire a mutator phenotype and also grew slower, consistent with our model (Supplementary Table S2).

We then tested additional quantitative predictions of our tug-of-war model between drivers and passengers. As the $H2AX^{+/-}$ tumors grew slower, their mutation rate should exceed the critical mutation rate that we predicted theoretically for the tug-of-war model between drivers and passengers (Supplementary Information). Indeed, the $H2AX^{+/-}$ tumors' mean mutation rate (28 Mb/y; 95% CI, 13–44) was approximately 10 times greater than human breast cancers (2.7 Mb/y; 95% CI, 2.3–3.2). The theory also predicts that more mutagenic and slowly growing cancers contain disproportionately more passengers that prevent accumulation of more drivers and hence slow down tumor growth (9). In fact, $H2AX^{+/-}$ tumors did not exhibit more drivers (Supplementary Table S2, $P > 0.5$, two-sided t statistic) than faster growing tumors, consistent with their suppressed accumulation by an overwhelming number of passengers (despite the higher mutation rate). Drivers were classified as amplified/deleted mouse homologs to the human oncogenes/tumor suppressors identified above (Supplementary Table S3). This analysis provides direct support for a tug-of-war between drivers and passengers, where passengers are as consequential as drivers, in an organismal environment during cancer development.

Effect of passengers on metastases in mice

Next, we asked whether passengers impacted not only primary tumor growth, but also metastatic development. Our theory

(Supplementary Information) indicates that passenger load is particularly detrimental for metastatic progression because it can both prevent and slow the growth of micrometastases. Because our transgenic mouse model does not metastasize, we used Her2-tranformed MCF-10A cells, with increasing passenger loads (see above), to investigate metastatic progression. These cells were (i) transfected with a luciferase reporter; (ii) injected into the tail vein of SCID female mice; and (iii) monitored for lung metastases *in situ* via thoracic bioluminescence. Metastases arose after week 4 (Fig. 4A) in all groups and grew faster in the unmutagenized strains, confirming our results. After 7 weeks, mice were sacrificed and the number of metastases in the mutagenized (10/20 nmol/L doxorubicin exposure) strains was 12-fold lower (95% CI, 9–19) than control groups, while aggregate metastatic load (bioluminescence) was 80-fold (95% CI, 54–128) lower (Fig. 4B and C). Thus, both the number of metastases and their average size declined with passenger load. Fewer observed metastases could arise because (i) fewer disseminating cells colonized/engrafted into new stroma; (ii) slower growth kept some metastases undetectable at 7 weeks; or (iii) passengers occasionally prevent the growth of already colonized cells. Because total burden was far less than would be expected from the cell line experiments, we believe that this second hypothesis cannot fully explain our observations. Our theory argues that this final possibility, unsuccessful progression of micrometastases, explains our observations. We did not, however, directly interrogate these three possibilities. The slower observed growth rates of metastases that we measured by direct bioluminescent monitoring also supports our hypothesis that passengers slow growth in a variety of microenvironments. Overall, passenger load dramatically reduces total metastatic progression and burden in our mouse experiments.

Discussion

Our findings indicate that accumulated passengers can be directly deleterious to cancer, by reducing cell proliferative fitness, significantly slowing down cancer growth, and impeding metastatic progression. Overall, we confirm several distinctive predictions of the deleterious passenger model that: (i) aggregate passenger load can overpower drivers and slow or prevent tumor progression; (ii) passengers have a deleterious fitness cost of approximately 0.1% to 1% per event; (iii) exceptionally high mutation rates inhibit carcinogenesis; and (iv) deleterious passengers can limit metastatic progression. These predictions were previously made using evolutionary simulations, theoretical analysis, and genomic analysis (summarized in the Supplementary Information; refs. 8, 9). In our new paradigm, genomic instability is a double-edged sword: It accelerates driver events, but eventually accumulates intolerable quantities of deleterious passengers.

Deleterious passengers can be a clinical diagnostic and a targeted phenotype. The direct anticancer effect of passengers that we found here can also explain how some of the most common chromosome-damaging chemotherapies work. Moreover, our findings suggest that increasing passenger load by genotoxic chemotherapies can have a more profound effect on tumors with initially higher passenger loads. This prediction is consistent with earlier findings (18, 19), as well as a recent and extensive pan-cancer study (20) that demonstrated significant reduction in mortality from cancers with the highest load of chromosomal alterations.

Recent studies also suggest that passengers trigger anticancer immune responses (14–17). This immunogenic role of passengers supplements and synergizes with the direct effects of passengers on cancer that we demonstrate here. Finally, our theory predicts that deleterious segregating (i.e., subclonal/private) of passengers should interfere with the acquisition of new drivers (43) and thus reduce genetic diversity (44, 45), which has been shown to be associated with drug resistance and survival both in our evolutionary modeling (8) and in pan-cancer clinical analyses (20). Therefore, deleterious passengers not only slow tumor progression, but also limit its mode of evolution, reducing the probability of resistance and preventing or slowing metastatic progression.

Accurately characterizing passenger load is essential to many clinical efforts. Quantifying passenger load for use as a genomic biomarker is challenging because the effect of individual mutations varies. We addressed this challenge by weighting passenger's impact by their length, copy number, and effect on genes and found that all of these factors are relevant. Overall, larger CNAs tend to be more deleterious, although this effect attenuates at very large sizes, and the deleterious effects of passengers are not confined to their effects on housekeeping genes. This is consistent with findings in natural populations where moderately deleterious mutations primarily cause cytotoxic stress via protein misfolding, disbalance, and aggregation (19, 46, 47), and not by abrogating essential gene function. Our quantitative measures accurately predicted experimentally measured fitness and cancer genomic patterns. Experiments directly studying whole-chromosome aneuploidy report similar findings: Aneuploid cells showed lower proliferative fitness (21). Here, however, we were able to develop a more quantitative and precise model of this effect (by virtue of our genotyping and study of shorter, focal CNAs). More ubiquitous and better genotyping methods (that characterize genomic rearrangements, SNMs in intergenic regions, epigenetic changes, and subclonal mutations) should improve our already useful measures of passenger load.

Therapeutics could therefore target cancer's deleterious passenger load by increasing passenger deleteriousness. Proposals to exacerbate passenger deleteriousness include (i) targeting essential cell functions lost by passenger deletions (48); (ii) increasing cytotoxic stress caused by passengers (19); (iii) increasing passenger load via DNA-damaging therapies (49); and (iv) neoantigenic immune therapies (14). Our experimental findings clinically benefit these efforts by (i) validating passenger's deleterious phenotype; (ii) identifying a biometric to direct such therapies; and (iii) suggesting that these treatments will work best at metastatic prevention and in conjunction with mutagenic therapies.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

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Other (principal investigator at Moffitt Cancer Center that directed the *in vitro* and *in vivo* contributions to this work): D.L. Morse

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