Identification of cancer sex-disparity in the functional integrity of p53 and its X chromosome network

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The disproportionately high prevalence of male cancer is poorly understood. We tested for sex-disparity in the functional integrity of the major tumor suppressor p53 in sporadic cancers. Our bioinformatics analyses expose three novel levels of p53 impact on sex-disparity in 12 non-reproductive cancer types. First, TP53 mutation is more frequent in these cancers among US males than females, with poorest survival correlating with its mutation. Second, numerous X-linked genes are associated with p53, including vital genomic regulators. Males are at unique risk from alterations of their single copies of these genes. High expression of X-linked negative regulators of p53 in wild-type TP53 cancers corresponds with reduced survival. Third, females exhibit an exceptional incidence of non-expressed mutations among p53-associated X-linked genes. Our data indicate that poor survival in males is contributed by high frequencies of TP53 mutations and an inability to shield against deregulated X-linked genes that engage in p53 networks.
cancer incidence and death rates are higher in males than females, and despite extensive genome-wide analyses (e.g., refs. 2,3), the explanation for this inequity is incomplete. Cancer sex disparity is evident across multiple non-reproductive, sporadic cancers. Male lifestyle has been blamed for greater exposure to carcinogens, however, it is likely that both environmental and genetic factors influence cancer incidence. Emerging molecular and physiological peculiarities that appear to affect disparity in animal studies, prompted NIH to demand preclinical evaluation in both males and females. Intriguingly, not all animals develop cancer, and in elephants, protection of both sexes is attributed to multiple copies of the *TP53* gene. The lack of cancer in elephants also argues that p53 cannot override an impact of hormones on cancer sex disparity. Critically, the single *TP53* gene copy is outstanding as the most commonly altered gene in human cancers. Further, in a compound mutant p53 mouse model that we generated, males developed more aggressive cancers and reduced lifespan than females. This triggered us to investigate the connection between p53 and cancer sex disparity and reduced lifespan than females. This triggered us to investigate the connection between p53 and cancer sex disparity.

Tumour suppressor p53 is a transcription factor that crucially integrates stress signals into protective cellular responses. Stresses such as DNA damage, trigger activating-modifications in p53, which promote temporary arrest to facilitate repair, or permanent growth inhibition, including senescence, apoptosis, and ferroptosis. P53 also targets core-regulatory molecules in metabolic pathways (glycolytic, lipid, and nucleic acid), immune responses, and senescence, apoptosis, and ferroptosis. Remarkably, the activities attributed to p53, include the four key processes recently identified through gene expression analyses to link to cancer sex disparity: (1) immune response, (2) apoptosis and cell cycle, (3) metabolism-related and (4) DNA-repair and p53-pathways (using standard autosome pathways of the gene set enrichment analysis (GSEA)). In healthy individuals, inherited genetic variants in the standard p53-pathways were more frequent than for all other pathways and linked to cancer exclusively, and not other diseases. These findings highlight the importance of p53 and its autosome partner proteins as the greatest natural deterrent against cancer. Whether these p53 functions are distinct between cancers in males and females remain unanswered.

P53 activities and expression are normally tightly regulated, and a universal signature of human cancers is the loss of effective p53 tumour-suppressor activity. Breakdown in p53 function is attributed almost equally to *TP53* gene mutation, or excessive levels of its key negative regulators: MDM2 and MDM4. *TP53* mutation not only strips away its tumour-suppressive capacities but also confers neomorphic, cancer-promoting properties, referred to as gain-of-function (GOF; reviewed). Discrepant rates of cancer and related mortality between males and females provoked us to investigate whether the tumour-suppressive ability of p53 is equally competent between the sexes. In this study of non-reproductive cancers, we identified three novel layers of risk for cancer sex disparity that are critically affected by p53 status and subject to the function and expression of its X-chromosome interactors.

### Results

#### Sex disparity in *TP53* mutation frequency in US population

The most common somatic cancers are more frequent in males. To explore this disparity, we examined whether mutation of the tumour-suppressor gene *TP53* differs in frequency in the human population, between male and female somatic cancers. This human sex-disparity study was prompted by our discovery of a link between p53-functionality and cancer aggression in male mice.

Directly measuring the relative general population cancer risk for *TP53* mutation between males and females is currently not feasible as: (1) it is an age-dependent parameter, which requires sampling across multiple age-brackets; (2) sampling every organ is impractical and (3) even with the advances in detecting circulating tumour DNA, the current technology lacks sufficient sensitivity.

Our alternative approach was to amalgamate two complementary data sets to compare *TP53* mutation incidence between male and female cancers. First, the National Cancer Institute’s Surveillance, Epidemiology, and End Results program (SEER data) provides the comprehensive cancer incidence for the overall US population. This cannot be obtained from the second data set, The Cancer Genome Atlas (TCGA). Rather, TCGA offers the rates of cancer-associated *TP53* mutations (or pathogenic mutations) from random cancer sampling across the sexes, but does not claim to be representative of population incidence. The pathogenic definition is built on the observation that most cancer-associated *TP53* mutations occur in its DNA-binding domain and cause interruption of p53 function (as defined in detail in the Methods section). From TCGA, we considered all *TP53* exome mutations with functional impact to be pathogenic. Specifically, we examined our hypothesis that *TP53* mutation frequency is distinct between the sexes by applying a probability calculation for 13 non-reproductive cancers. Explicitly, this represents all the cancers with at least five TCGA cases in all four groups: female mutant *TP53*, female wild-type (wt) *TP53*, male mutant *TP53* and male wt *TP53*. Our calculations show for the first time that males have a higher probability of developing cancers with *TP53* mutations than females, for the majority of non-reproductive cancers: subsequently referred to as the 12 disparity cancers (Fig. 1a; Table 1; Supplementary Table 1). This finding forms the focus of this study. Kidney (KIRC), which has a very low *TP53* mutation incidence and originates from distinct developmental tissue, indicates that our finding cannot simply be explained as an obvious direct correlation between cancer prevalence and *TP53* mutation.

To examine the functional impact of *TP53* mutations in the TCGA cohort, we analysed TCGA for overall cancer patient survival, for the 12 disparity cancers across 14 years. Together, patients with mutated *TP53* from these 12 disparity cancers had significantly poorer survival outcomes than their wt *TP53* counterparts (Log-rank test; *p*-value = 0.0002, Fig. 1b). It is relevant to add that this finding was validated recently in a study of individual cancers, where nine of the cancers in this study showed poorer survival for patients with *TP53* mutation, while individual data analyses for the other three cancers were not included. Given our finding that *TP53* mutations occur at higher frequency in males than in females in the 12 disparity cancers, we expect *TP53* mutational status to contribute significantly to the higher incidence of cancer deaths in males in the general population, over females.

This defines our first cornerstone that: *TP53* mutation frequency is higher among US males than females for the majority of non-reproductive cancers, corresponding with poorest survival outcomes for these cancers.

### X chromosome encodes many proteins that interact with p53

The normal strict control of the tumour suppressor p53 and its multiple downstream effector pathways (reviewed in ref. 23) are disrupted in cancer, either by *TP53* mutation or by malfunction in this p53 network. The possibility that the p53 network is not equally functional between males and females is unexplored. We chose to search for connections between p53 and the X chromosome. The p53 network is not equally functional between males and females is unexplored. We chose to search for connections between p53 and the X chromosome.
chromosome because: (1) numerous tumour-suppressor genes are encoded on the X chromosome, but are under-studied for connection to p53 and sex disparity; (2) X-chromosome genes are not yet incorporated into established p53 networks (eg. KEGG); (3) X-chromosome analysis is frequently avoided in sporadic diseases because X-inactivation (Xi) silencing in females complicates data interpretation (reviewed in refs.27,28) and (4) males are at greater disease risk than females from X-chromosome mutations (reviewed in ref.29).

To identify coding genes that interact with p53 on the X chromosome, we adopted the unbiased Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) database tool. A reproducible p53-STRING set of 90 genes was defined (with confidence limits ≥ 0.3) from among the ~800 coding genes on the X chromosome (Supplementary Data 1, TAB1; Supplementary Fig. 1a). We note that due to the rapid pace of new discoveries, some partnerships remain to be captured. Among the STRING set are key p53 modifiers (kinases, E3-ligases and deubiquitinases). Gene Ontology (GO) analysis shows that the p53-STRING set is rich in fundamental p53 regulatory processes, including regulation of apoptosis, cell cycle, DNA structural integrity and hypoxic response. Additional notable processes included are regulation of protein acetylation and histone modifications, among others (Supplementary Fig. 1b; with analyses shown for both the inclusion, or not, of TP53, respectively, Supplementary Data 1, TAB2, 3). The specific example of the p53-negative regulator HUWE1 supports this link, where reduction of its levels corresponds with diminished cell growth only in cells with wt TP53, but not mutant (among 135 lines of the Achilles project, Supplementary Fig. 1c); consistent with the relief of wt p53 from constraints resulting in growth inhibition.

**Fig. 1** The frequency of cancer with TP53 mutation is greater for males than females in multiple non-reproductive cancer types with TP53 mutation corresponding to poorest survival. a Predicted TP53 mutation incidence in male and female cancers in the US population as calculated from combined SEER and TCGA data for the 13 most abundant non-reproductive cancers: oesophageal carcinoma (ESCA), liver hepatocellular carcinoma (LIHC), bladder urothelial carcinoma (BLCA), head and neck squamous cell carcinoma (HNSC), lung squamous cell carcinoma (LUSC), skin cutaneous melanoma (SKCM), lower grade glioma (LGG), rectum adenocarcinoma (READ), stomach adenocarcinoma (STAD), colon adenocarcinoma (COAD), pancreatic adenocarcinoma (PAAD) and lung adenocarcinoma (LUAD and the exception (demarcated by a dashed box), kidney renal clear cell carcinoma (KIRC). b Overall survival (OS) across 14 years, for patients from the 12 disparity cancers in TCGA is stratified for cancers with wt TP53 (aquamarine) and mutant TP53 (turquoise).
X-chromosome exome mutations are abundant in male cancers. To search for evidence of sex disparity in the p53-STRING set, we analysed TCGA data for somatic exome mutations (DNA) and their expression (mRNA); as outlined in Fig. 2a (with details included in the Methods section). To adjust for potential confounding factors in our statistical tests, we applied propensity scores to the analyses as described by Yuan et al.2. Patient data were controlled for the key parameters of age, race, smoking history and tumour stage (as detailed in the Methods). At the whole-exome level, only minor differences in the total number of gene mutations per patient (determined for 1733 females and 2757 males; using the Poisson log linear model, 95% CI [1.01, 1.02], p-value ≈ 0) were evident between the sexes in the disparity cancers, excluding the Y chromosome (Fig. 2b). In contrast, separate analysis of the shared allosome, the X chromosome, revealed a higher incidence (≈ twofold) of exome mutations in cancers of females than males (95% CI [1.86, 1.92], p-value ≈ 0; Supplementary Data 2, TAB1). This was also evident for the p53-STRING set for the disparity cancers together (95% CI [1.55, 1.72], p-value ≈ 0; Supplementary Data 2, TAB1–7; and shown also for each cancer individually in Supplementary Fig. 2b). This is in accord with there being two X chromosomes in females, and one in males per chromosome set. Xi during development of a single female X chromosome ensures single-allele expression of most X-linked genes in females. This effectively evens X-chromosome gene expression between the sexes. Exceptions to this in females are the limited ~15% of escape-genes32, which are largely pseudoautosomal (reviewed in ref. 33) with a few exceptions3. The identity of the X-chromosome copy that is silenced by Xi cannot be deduced from TCGA exome data. Equally, the X-chromosome gene mutations that are expressed cannot be identified from exome sequencing. To identify which X-chromosome exome mutations were expressed in male and female cancers, it was necessary to search for the corresponding mutations in their mRNA.

Elevated X-chromosome RNA-mutated allele frequency in males. Variant allele frequency (VAF) is a commonly used metric employed to identify somatic variants at the DNA level. To date, a measure for RNA expression of DNA mutations has not been formally assigned. To fill this gap, we created a novel concept: RNA-mutated allele frequency (RMAF), which we define as the mRNA equivalent of VAF. Explicitly, RMAF is a measure of the relative frequency with which a mutation in DNA is expressed in mRNA, relative to the total expressed mRNA (refer to Methods).

We predicted that for the X chromosomes, the RMAF of females would be lower than for males. This was founded on the expectation that for males, all mutations from their single X chromosome can be expressed. For healthy females, expectation of lower RMAF was founded on the prediction that mutations are evenly spread across the two individual X chromosomes, and that X inactivation is completely random. However, in contrast to healthy females, in cancers we could not presume that this would be the case. This led us to formerly test RMAF across the 12 disparity cancers for females and males.

We found that in female cancers, more than half of their X-chromosome exome mutations were not transcribed into their corresponding mRNA (4002/7416; 54% RMAF = 0, median 0).

### Table 1 Frequencies of cancers and TP53 mutation by sex.

| Cancer type | Sex | Total patients by disease (TCGA) | Patients with TP53 mutation (TCGA) | Frequency of TP53 mutation per cancer (TCGA) | Frequency of cancer per 100,000 (SEER) | Frequency of TP53 mutation per 100,000 (SEER & TCGA) | Ratio: M/F frequency of cancer with TP53 mutation in the US population |
|-------------|-----|---------------------------------|-----------------------------------|-----------------------------------------------|---------------------------------------|------------------------------------------------|---------------------------------------------------------------------|
| ESCA        | F   | 27                              | 19                                | 0.70                                          | 1.7                                   | 1.2                                           | 5.5                                                                |
|             | M   | 158                             | 137                               | 0.87                                          | 7.6                                   | 6.6                                           |                                                                     |
| LIHC        | F   | 121                             | 26                                | 0.21                                          | 4.3                                   | 0.9                                           | 4.9                                                                |
|             | M   | 252                             | 89                                | 0.35                                          | 12.7                                  | 4.5                                           |                                                                     |
| BLCA        | F   | 108                             | 56                                | 0.52                                          | 8.7                                   | 4.5                                           | 3.9                                                                |
|             | M   | 303                             | 147                               | 0.49                                          | 35.8                                  | 17.4                                          |                                                                     |
| HNSC        | F   | 138                             | 98                                | 0.71                                          | 6.3                                   | 4.5                                           | 2.7                                                                |
|             | M   | 371                             | 267                               | 0.72                                          | 16.5                                  | 11.9                                          |                                                                     |
| LUSC        | F   | 129                             | 102                               | 0.79                                          | 7.4                                   | 5.9                                           | 2.6                                                                |
|             | M   | 362                             | 323                               | 0.89                                          | 17                                   | 15.2                                          |                                                                     |
| SKCM        | F   | 179                             | 23                                | 0.13                                          | 16.8                                  | 2.2                                           | 2.2                                                                |
|             | M   | 286                             | 48                                | 0.17                                          | 28.2                                  | 4.7                                           |                                                                     |
| STAD        | F   | 157                             | 74                                | 0.47                                          | 5.3                                   | 2.5                                           | 2.0                                                                |
|             | M   | 280                             | 139                               | 0.50                                          | 10.1                                  | 5.0                                           |                                                                     |
| LGG         | F   | 227                             | 98                                | 0.43                                          | 5.5                                   | 2.2                                           | 1.7                                                                |
|             | M   | 281                             | 141                               | 0.50                                          | 7.2                                   | 3.6                                           |                                                                     |
| READ        | F   | 77                              | 57                                | 0.74                                          | 9.7                                   | 7.2                                           | 1.7                                                                |
|             | M   | 89                              | 70                                | 0.79                                          | 15.3                                  | 12.0                                          |                                                                     |
| COAD        | F   | 215                             | 114                               | 0.53                                          | 27.4                                  | 14.5                                          | 1.4                                                                |
|             | M   | 234                             | 138                               | 0.59                                          | 33.5                                  | 19.8                                          |                                                                     |
| PAAD        | F   | 79                              | 51                                | 0.65                                          | 11                                   | 7.1                                           | 1.3                                                                |
|             | M   | 102                             | 66                                | 0.65                                          | 14                                   | 9.1                                           |                                                                     |
| LUAD        | F   | 277                             | 143                               | 0.52                                          | 17.4                                  | 9.0                                           | 1.3                                                                |
|             | M   | 239                             | 129                               | 0.54                                          | 21.7                                  | 11.7                                          |                                                                     |
| KIRC        | F   | 179                             | 5                                 | 0.03                                          | 10.8                                  | 0.3                                           | 3.1                                                                |
|             | M   | 314                             | 5                                 | 0.02                                          | 21.3                                  | 0.3                                           |                                                                     

Table 1 frequencies of cancers and TP53 mutation by sex.

- M male, F female
- TCGA data22
- SEER data21
- SEER data for oral cavity and pharynx
Given the lower incidence of non-reproductive cancers in females, we hypothesised that they are preferentially protected from the expression of cancer-risk gene mutations, compared with males. As we were unable to test this in healthy people, we examined data from cancer biopsies, aware that we were searching for remnants of a broken protective system. We compared the expression of X-chromosome mutations between cancers of females and males, where X-chromosome exome mutations that were not detected at the mRNA level (RMAF ≤ 0.20; Fig. 3) are referred to as non-expressed mutations (NEMs, see the Methods section).

The incidence of NEMs reached significance only in females (Fig. 4). In total, over half of females (268 genes (~1/3 of the X-linked coding genes) were significantly less expressed in cancers of females than males. GO analysis of these 268 genes identified pathways of protein acetylation (including histone acetylation); histone modification and negative regulation of both organelle organisation and microtubule depolymerisation (Supplementary Data 3, TAB1–3; Supplementary Fig. 3a). If the double load of X-chromosome exome mutations in females (Supplementary Data 2) were equally distributed across the active and inactive X-chromosome genes on a random basis, a greater number of NEMs would be expected in female cancers distributed as a function of gene size, simply due to Xi. Importantly, we did not find significant correlation between X-gene sizes and NEMs (Pearson coefficient: \( r = 0.3 \), data not shown).

Strikingly, our analysis revealed an unexpectedly high incidence of NEMs in the p53-STRING gene set (Wilcoxon rank test \( p\)-value = 0.001 Supplementary Data 3, TAB4, 5). The most significant NEMs in these cancers occurred in the p53-STRING genes FLNA, MED12, HUWE1, ATRX (Fig. 4). In total, over half the p53-STRING genes (50 out of 90) were identified with a significantly disproportionate NEM incidence between males and females (adjusted \( p\)-value ≤ 0.05). GO analysis of these 50 genes identified their involvement in fundamental pathways: histone modifications (ubiquitination, H3–H9 modification...
genes between females and males of the 12 disparity cancers. P53-STRING set genes are blue, others are gold. NEMs showing a significant acetylation and covalent chromatin modification were prevalent in processes associated with internal amino acid metabolism (Fig. 4). In females, EM genes showed a significantly lower proportion of their X-linked gene mutations compared with males (Supplementary Fig. 4a, b). The functional association of these 50 genes with p53 was confirmed by extensive literature mining (Supplementary Data 3, TAB6; Supplementary Fig. 3b). The functional association of these 50 genes with p53 was confirmed by extensive literature mining (Supplementary Data 3, TAB7). Notably, analysis of the KIRC data set did not identify significant NEMs for the X chromosome (Supplementary Data 3, TAB8). However, analyses of larger KIRC data sets will be required for validation. Overall, this exceptional incidence of NEMs among these p53-STRING genes supports our hypothesis that resistance to cancers of the disparity set is conferred in females through selective protection of the p53-X-chromosome network. While NEMs are relevant to cancer protection, it is the expressed mutations that pose cancer risk.

This defines our third cornerstone that compared with males, females express a significantly lower proportion of their X-linked gene mutations, and this is outstanding for the p53-STRING set.

**Frequent expression of X-linked gene mutations in cancers.** In male and female cancers, X-chromosome expressed mutations (EMs, RMAF ≥ 0.75, see Methods) were numerous (Supplementary Data 4, TAB1). Consistent with the relevance of the p53-STRING set in cancer, these genes were among those with the most frequent EMs. Specifically, more than half of the p53-STRING set genes (47) were among the 299 genes with EMs in female cancers (Supplementary Data 4, TAB2,3). Similarly, in male cancers, 53 genes of the p53-STRING set were among the 341 genes with EMs (Supplementary Data 4, TAB4,5). This abundance of p53-STRING set EMs in males and females supports the disruption of p53 networks in cancers of both sexes (Supplementary Data 4, TAB6). GO analysis of the EM genes in females (299) and males (341) identified distinct pathway vulnerabilities between the sexes (Supplementary Fig. 4a, b, respectively; Supplementary Data 4, TAB7). In females, EM genes were prevalent in processes associated with internal amino acid acetylation and covalent chromatin modification, while in males the processes of glycosyl compound metabolic process, histone modification and response to iron(III) ion were pronounced.

**X-linked inhibitors of wt p53 are highly expressed in cancer.** To examine whether altered gene expression levels of the p53-STRING set are evident in the disparity cancers, we adopted the comparative expression analysis tool GSEA. This computational approach was applied to measure statistically significant differences in the expression of a designated list of genes, between two related populations. Enrichment in gene expression was quantified according to a normalised enrichment score (NES), and statistical significance was defined by an adjusted p-value of <0.25.

We chose to focus on the X-linked negative regulators of wt p53. We postulated that high expression of these negative regulators could reduce wt p53 activity, in an analogous manner to high levels of the MDM proteins, which have proven to be oncogenic (reviewed in ref. 34). Literature mining led us to identify 11 negative regulators of wt p53 among the p53-STRING set: UBE2A (RAD635), MAGEA236 and UTP14A37, CDK1638, PPEF139, HUWE140, CUL4B41, DDX5342, NOX143, PMSD1044 and TAF145,46. We tested the relative expression levels of these genes in tumours and their matched normals, for each cancer type separately, with additional stratification according to wt TP53 status and patient sex. We undertook these studies on 11 of the 12 disparity cancers, but excluded LGG, due to a lack of matched normal tissue.

Expression of these genes was evidently higher in all the 11 cancers compared with their matched normals (Fig. 5a displays graphically the results of the analyses that are recorded in Supplementary Data 5, TAB1). Notably, this correlation was significant in males for seven of the cancers and for six in females. Even where significance fell below the designated threshold, there was a universal trend for enhanced expression in the tumours over the normals. It is appropriate to emphasise that this analysis ranks the expression of each gene individually for each cancer type.
TP53 biological outcome of survival in cancers with wt

We tested whether individual expression of TP53 affected survival in cancers with wt TP53 and their matched normals, we note that these may be affected by the existence of cancer cells and/or by adjacent tissue. In the matched normals, KDM6A, DDX3X and UBA1 were significantly differentially expressed among the TP53-STRING set.

Sex disparity of p53-STRING gene expression in normal adults. We next examined the gene expression levels between males and females of the X-linked p53-STRING set in tissues classified as normal and discriminated as cancer-free, from the TCGA cohorts across all the disparity cancers. While these are referred to as matched normals, we note that these may be affected by the existence of cancer cells and/or by adjacent tumor tissues in these patients (Supplementary Data 5, TAB2). In the matched normals, KDM6A, DDX3X and UBA1 were significantly differentially expressed among the p53-STRING set.

For comparison, we extended this test to the healthy samples of the 1000 genomes project, which are lymphoid in origin. Among these, statistically significant differential expression (DE) was identified between the sexes for 13 of the p53-STRING set genes (among the 95 X-linked DE genes with adjusted \( p \)-value \( \leq 0.05 \), Limma, Benjamini–Hochberg adjustment). All 13 genes were expressed at higher levels in females than in males (Supplementary Fig. 5; Supplementary Data 5, TAB3) with KDM6A, DDX3X and UBA1 prominent, which complements our findings in the normals matched to the tumours.

Discussion

The well-documented, higher incidence of lethal, non-reproductive cancers in males than females is attracting a growing interest into its underlying causes (eg., refs.2,3), however, the picture is far from clear. Guided by our previous study demonstrating sex disparity in a compound mutant p53 mouse model8, together with the unprecedented incidence of TP53 mutations in human cancers, we tested the contribution of p53 to cancer sex disparity. We identified three levels of connection between p53 and sex disparity in somatic non-reproductive cancers. For the first time, our statistical analyses predict a significantly higher frequency of cancers with mutated TP53 in males than females, for 12 common sporadic cancers, across the US population (as determined through the integration of TCGA and SEER data). The exception is KIRC, which was more common in males than females, but with exceptionally few TP53 mutations. Notably, in KIRC, although TP53 mutation is infrequent, poorest survival
corresponds with high levels of MDM2, which emphasises the importance of evading p53 tumour suppression also in this disease. This outlier dismisses a direct correlation between cancer prevalence and TP53 mutation as its explanation. These findings are in line with somatic TP53 mutations accumulating preferentially in tissues arising from the embryonic layers of the ectoderm and the endoderm, in contrast to germline TP53 mutations which at least for non-reproductive cancers, preferentially occur in tissues derived from the mesoderm, which includes the kidney.

It is pertinent to our study that the ethnic population composition of TCGA and SEER correlate remarkably closely. The verified TCGA, SEER proportions are for Whites ~73%: ~78%, respectively, for Black or African Americans ~8%: 13%, respectively, and are near equivalent for Asians. These very similar proportions are relevant as TP53 mutation frequencies appear lower in Caucasians for certain cancer types (reviewed in ref. 49). It is also relevant to note that TCGA is comprised of primary disease, regardless of whether originating from resections or biopsies, sampled from metropolitan centers. SKCM is the only exception, but most of these tumours are wt TP53. The nature of these samples may have contributed to an underestimate of TP53 mutation incidence in the population, as metastatic disease is frequently associated with mutation of this tumour suppressor (reviewed in ref. 51) and non-metropolitan disease is frequently more severe. In sum, the incidence of TP53 mutation in the US population may be even higher than our calculations predict, with males at greater risk than females.

Our findings indicate that tissue-specific vulnerability to somatic TP53 mutation is exacerbated by patient sex. This is in line with the specific instance of glioblastoma astrocytes, in which components of the p53 and RB pathways were recently reported to contribute to sex disparity. This could result from a number of causes. One possibility is that male non-reproductive tissues (pre-cancer) may be more exposed to carcinogens and/or prone to spontaneous pathogenic TP53 mutation than their female counterparts. Alternatively, females may have a better tumour-suppressive capacity due to a greater ability to clear or restrain cells with TP53 mutations, compared with males. Testing these hypotheses will require prospective sampling for TP53 mutations in large data sets from healthy populations, across multiple tissues, with temporal follow-up. Currently, this information is unavailable. Significantly, among the disparity cancers, the occurrence of TP53 mutation corresponded with the worst survival outcomes. This suggests that TP53 mutation is an overriding oncogenic event. Indeed, many of the regulatory and auto-regulatory loops with p53 are operative only in the context of wt p53. As males have higher cancer incidence and as we demonstrate, a greater frequency of TP53 mutations than females, this is a likely contributor to their poor cancer survival statistics. While this study is restricted to the US population, similar statistics would be expected across the western world.

The second major level of contribution of p53 to sex disparity is through its connection to the X allosome. While females have two chromosomes, developmental Xi reduces expression close to that of the single X chromosome in males. Females are protected from germline mutations by Xi and mosaicism (reviewed in ref. 50). Accordingly, X-linked inherited diseases manifest predominantly in males, frequently at a young age. Notably, with our colleagues we recently identified that p53 drives Xi in females during development, and its absence in females is fatal, largely associated with neural tube defects. In contrast, most cancers arise from somatic mutations in the aging population, where we predicted alternative mechanisms must account for lower cancer incidence in females. We uncovered a novel p53-X-chromosome network (Supplementary Data 1), with cancer protection potential that is particularly advantageous for females. Generally in cancers, we identified that somatic X-linked gene mutations, including the genes of the p53-STRING set, occur twice as frequently in females as in males, reflecting their distinct X-chromosome copy numbers. However, this does not correlate with cancer incidence. In contrast, we identified that our newly identified X-linked p53-STRING gene set are at peculiar risk of deregulation in male cancers.

The functional relevance of this p53-STRING set emerged though studying the mRNA levels of these genes. Expression analysis (by GSEA) of the 11 p53-STRING set genes reported to negatively regulate p53, exposed a higher level of expression in cancers with wt TP53, than in the matched normal tissues (Fig. 5a). This proved to be statistically significant in seven cancers in males and six in females, although for all cancers this trend was evident. Survival analysis examining high expression levels of three of these genes (UBE2A, MAGEA2 and UTP14A) corresponded with poorer survival in five different cancers (Fig. 5b). These findings align with compromised wt p53 activity, through enhanced levels of its negative regulators posing a major cancer risk to TP53.

As each cancer develops along a different trajectory of mutations, we did not expect that all cancers would be impacted equally by these genes. It is expected that in different cancer types and for each individual cancer case, different combinations of genes will be involved, with different permutations of deregulated expression and/or mutations. A particular permutation would be influenced by the status of TP53, whether these X-linked genes are either positive or negative regulators/effectsors, and further whether these genes themselves have undergone mutation.

An interesting translational angle arises from this data. Wt TP53 status is generally a good prognostic marker for response to conventional genotoxic drugs due to their reliance on wt p53 activation (reviewed in ref. 60). In the context of high expression of negative regulators of wt TP53, as exposed in our study, p53 activation is likely to be ineffective, leading to poor response, and consequently to poor survival. Our findings suggest clinical pertinence, where altered protein expression of negative p53 regulators such as these offer potential value as prognostic markers (as suggested for UBE2A for liver cancer61 and UTP14 for colorectal cancer62 and MAGEA2 for lung cancer63). Also when overexpressed, these genes represent targets for therapy in these male dominated cancers. A specific example is HDACi trichostatin A plus etoposide administered to treat cancers with elevated MAGEA247. Our study lays a foundation for developing rational strategies to treat cancer patients on the basis of expression levels of these regulators in the context of TP53 mutational status. The greater prevalence of these cancers in males, predicts disproportionate application of these tools for prognosis and treatments between the sexes.

In contrast to these cancer risks, in females, both in the cancer-matched normal TCGA samples and the 1000 genomes project, we identified elevated expression of a number of p53-STRING set genes that are noted for tumour-suppressive capacity. Their higher levels in females are predicted to afford them a more robust innate cancer protection compared with males, who were identified with inherently lower expression. The most prominent of these are candidate tumour suppressors; KDM6A (lysine demethylase 6 A), DDX3X (dead-box RNA helicase 3) and UBA1. Notably, KDM6A inhibits EMT by antagonising TGF-β induced genes, which are instrumental in this process. At the other extreme, elimination of KDM6A is proposed as an independent marker of prognosis for pancreatic cancer65. DDX3X protects against replication stress through its regulation of DNA damage repair66. Pertinently, a peculiar male vulnerability to DDX3X mutation has just been reported for melanoma67.
that male and female cancers are likely to benefit from treatment options, cancer surveillance and prevention. While sex discrepancy in treatment efficacy is addressed for a variety of cancers, the gender gap in cancer risk is still substantial. Poorer survival outcomes for patients with cancer sex disparity. First, the frequency of males with lung, prostate, and colorectal cancer is higher than in females. Secondly, the higher frequency of females with breast and endometrial cancer is observed. A third level is peculiarly vulnerable to the consequences of X-linked gene mutations, particularly for the p53-STRING set genes among the disparity cancers, across the 12 disparity cancers, normalised gene expression and survival data from the TCGA data set were accessed using The Broad Institute GDAC Firehose and analysed in R. Kaplan–Meier survival curves were generated using the top and bottom medians of gene expression, and differences in survival distributions between medians were tested using a log-rank test.

**Methods**

**Data source and propensity scores.** TCGA patient data were accessed using the The Broad Institute GDAC Firehose79. Initially, propensity scores based on patient sex as the independent variable. Patients with available sex annotation and <15,000 somatic mutations were analysed.

**Exome mutations, RMAF and mutation classification.** For each non-reproductive cancers, a full list of exome level somatic mutations was downloaded from Genomics Data Commons Data Portal and GDAC Firehose, more precisely, the Oncotator21; annotated mutations were analysed. Mutations classified as "Nonsense_Mutation", "Splice_Site" and "Frame_Shift_Type" were used to focus on mutations with the potential of having a more profound functional impact. Patients with available sex annotation and <15,000 somatic mutations were analysed. To assess whether DNA mutations in the X chromosome were transcriptionally and translationally silenced, we next classified males and females into three subgroups: NEMs, EMs, and NEMs plus EMs (NEM/EM). For each cancer type, we combined the exome somatic mutation list with the corresponding TCGA RNAseq BAM files using the Cancer Genomics Cloud platform by Seven Bridges (http://www.cancergenomicscloud.org/). For each X-chromosome DNA mutation, we defined a new metric: the DNA-mutated allele frequency (RMAF), an equivalent to variant allele frequency (VAF) commonly used to call mutations at DNA level (Supplementary Figs. 6, 7). RMAF was calculated by piling all overlapping mRNA reads at the genomic position, where the DNA mutation was called. When a sample had multiple somatic DNA mutations present in the same gene, only the mutation with the highest RMAF was used. When mRNA reads were not available on the TCGA (e.g., RNA-seq), the genomic position for a mutation, we calculated RMAF the following way: firstly, we checked if the gene in question was generally expressed in the corresponding cohort (male or female; with at least five counts per million (CPM) in the majority of samples), no expression was found, the mutation was discarded. Secondly, we calculated a z-score based on the gene expression of the corresponding sample relative to its corresponding cohort (male or female). If the expression of the gene was substantially diminished (i.e., 4 standard deviations below the mean; z-score ≤ −4) we concluded that the mutation had a functional effect in that sample (a knockdown effect) and assigned a RMAF of 1. Otherwise (z-score > −4), we discarded the mutation as its impact cannot be assessed. When mRNA reads were sufficiently abundant, RMAFs were calculated using Python and the Pysam library (https://github.com/pysam-developers/pysam), a wrapper of hislib and samtools82. Based on the relative RMAF distributions in the males and females, we established two thresholds in an effort to account for tumour purity and heterogeneity and classified mutations with an RMAF greater or equal to the threshold of 0.75 as EMs and those less or equal to 0.2 as NEMs.

**NEMs, EMs and the X chromosome.** After quantifying NEMs and EMs, we applied the propensity score algorithm and tested for significant differences in NEMs and EMs numbers between male and female patients. We applied a weighted chi-squared test and highlighted genes that had adjusted p-values ≤ 0.05 (Benjamini–Hochberg adjustment). We calculated a statistic for each of the 561 genes with NEMs, using the propensity score weighting which, under the null hypothesis: no association between a patients’ sex (female vs male) and no association with the status of the expression of the specific gene mutation (expressed versus not) would be asymptotically distributed as chi-squared on one degree of freedom. By combining these statistics across all the genes and test for differences in the distribution of sex differences between the p53-STRING set and each X-chromosome gene, we collected the associated chi-scores: the signed square roots of the chi-squared statistics, with the sign determined in the same way for all genes. We assigned a
plus sign if the proportion of NEM in females was larger than the proportion of NEM in males, and the converse was discriminated with a minus sign. These chi-scores should be asymptotically distributed as standard normal (mean 0, standard deviation 1) under the null hypotheses, for both the p53-STRING set and other genes. We tested (Wilcoxon rank test) the difference in distribution between these two groups and found that p53-STRING set genes host significantly larger number of NEMs compared with other genes in the X chromosome.

Differential expression. Raw gene-level counts for each cancer type were downloaded from The Broad Institute GDAC Firehose. Reads were converted to counts per million (CPM). Lowly expressed genes (fewer than 5 CPMs) were discarded. CPM counts were adjusted and normalised by library size using the R package edgeR. By accessing the clinical data and mutation lists, two groups were analysed: Wi (TP53 Tumours) and Mt (TP53 tumours) (all samples with pathogenic TP53 mutations), each one stratified by patient sex. In addition, RNAseq count data from 465 healthy lymphoblastoid cell lines (derived from normal bloods) was used for enrichment analysis. We ignored genes that sorted into a class with a fold change $\leq 2$.

Gene set enrichment analysis. To further investigate the role of p53-STRING genes in the disparity set, after performing differential expression analysis (normal vs. wt TP53 tumours), the corresponding gene lists ranked by fold change were analysed for enrichment of negative X-linked p53 regulators using the fgsea package in R. Normalised enrichment scores (NES) and adjusted p-values (adjustment by FDR) were used to highlight the enrichment of these genes in wt vs. tp53 tumours.

ClueGO cytoscape analysis. Gene Ontology (GO) analysis of the gene sets was performed in ClueGO Cytoscape. The following parameters were selected: GO tree interval between levels 3 and 8; GO terms with at least three proteins and 4% of proteins; a kappa score of 0.2 and only GO terms with a false discovery rate of less than 0.2 were included in the enrichment analysis.

STRING analysis. Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) database tool was adopted for analysis of X-linked genes that link to TP53 (version 10.5; https://string-db.org/cgi/network.pl?taskId=PpCASpZQ8dUs). TP53 was coincidently included in the gene input, to define association. Parameters of selection included: textmining, experiments, co-expression, gene-fusion, and co-occurrence. We ignored genes that sorted into a confidence score $< 0.3$.

Data availability. The data generated and analysed during this study are included in this published article and its supplementary information files.

Code availability. Code for normalisation, differential expression, log ratios tests, survival analysis, propensity scores calculation and RMAF calculation is available at https://github.com/fcarania.

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Author contributions

The study was conceived and the research designed by S.H., F.C. and Y.H. Bioinformatic analyses were undertaken by F.C. S.H. undertook data analyses. Biostatistics were undertaken by T.P.S. and A.H. H.C. and H.L. contributed scripts. T.S. provided the data. The paper was prepared by S.H., F.C. and Y.H. with input from G.L. and the other authors.

Competing interests

The authors declare no competing interests.
