Unequal prognostic potentials of p53 gain-of-function mutations in human cancers associate with drug-metabolizing activity

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Mutation of p53 is the most common genetic change in human cancer, causing complex effects including not only loss of wild-type function but also gain of novel oncogenic functions (GOF). It is increasingly likely that p53-hotspot mutations may confer different types and magnitudes of GOF, but the evidences are mainly supported by cellular and transgenic animal models. Here we combine large-scale cancer genomic data to characterize the prognostic significance of different p53 mutations in human cancers. Unexpectedly, only mutations on the Arg248 and Arg282 positions displayed significant association with shorter patient survival, but such association was not evident for other hotspot GOF mutations. Gene set enrichment analysis on these mutations revealed higher activity of drug-metabolizing enzymes, including the CYP3A4 cytochrome P450. Ectopic expression of p53 mutant R282W in H1299 and SaOS2 cells significantly upregulated CYP3A4 mRNA and protein levels, and cancer cell lines bearing mortality-associated p53 mutations display higher CYP3A4 expression and resistance to several CYP3A4-metabolized chemotherapeutic drugs. Our results suggest that p53 mutations have unequal GOF activities in human cancers, and future evaluation of p53 as a cancer biomarker should consider which mutation is present in the tumor, rather than having comparison between wild-type and mutant genotypes.

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The TP53 gene, which resides on chromosome 17p13.1 and encodes the p53 protein, is the most frequent target for mutation in human cancers, with over 50% of all observed tumors exhibiting mutation at this locus.1,2 The tumor suppressive functions of wild-type p53 are often explained by its transcriptional functions in the nucleus.3 In response to oncogenic mutations or DNA damage, p53 triggers a transcriptional program of cell cycle arrest, DNA repair, senescence, apoptosis4,5 and metabolic remodeling6 (Figure 1a). The vast majority (95%) of p53 mutations in human cancers are missense mutations, sitting within the DNA-binding domain (amino acids 102–292) with hot spots at codons R175, Y220, G245, R248, R249, R273 and R2827 (illustrated in Figure 1b). A well-established classification system for p53 mutation is based on its roles as a transcriptional factor. Mutations that occur in the p53-DNA-binding surface are defined as contact mutations (R273H and R273K) and mutations that cause conformational instability of the p53 protein are classified as a structural mutations (R175H, Y220C, G245S, R248Q, R249S, R282W, etc)8 (illustrated in Figure 1b).

In addition to its transcription-dependent functions, the p53 protein has also been found to function in mitochondria,9 where it binds to Bcl-XL and induces mitochondrial outer membrane permeabilization (MOMP) and apoptosis.10 According to a p53–Bcl-XL complex model determined by NMR chemical shift perturbation, the p53-hotspot mutations R248W, R248Q and R282W sit in the binding surface toBcl-XL9,11,12 (Figure 1c). It is currently unknown if these ‘Bcl-XL contact’ mutations confer any specific cancerous phenotypes.

It is becoming increasingly evident that mutant p53 proteins not only lose their tumor-suppressor function but some acquire oncogenic gain-of-function (GOF).1,7,13,14 The most compelling support for GOF comes from mice engineered to harbor some of the hot spot tumor-associated p53 mutations. When compared with heterozygous or null (p53+/− or p53−/−) mice, animals with one mutant allele equivalent to human R175H or R273H showed more spontaneous carcinomas, sarcomas and lymphomas. Although these mutants increased tumor metastasis, they do not shorten mouse survival time as compared with p53-null background.15,16 It was also found that R248Q mutation could accelerate onset of all tumor types

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Abbreviations: LOF, loss-of-function; GOF, gain-of-function; MOMP, mitochondrial outer membrane permeabilization; TCGA, The Cancer Genome Atlas; CCLE, Cancer Cell Line Encyclopedia; GSEA, Gene Set Enrichment Analysis; FOLFIRI, folinic acid-fluorouracil-irinotecan regimen; IC50, half maximal inhibitory concentration; CRC, colorectal cancer; GEO, gene expression omnibus

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and shorten the survival of mice, thus suggesting that hotspot mutants may confer different type and magnitude of GOF effects. A recent in-depth study demonstrated that R273H mutant gains new function by activation of EGFR signaling via suppressing mir-27a expression. It is of great importance to clarify the prognostic significance of p53 mutations, as genes that display oncogenic activities may present useful information for cancer prognosis. Presumably owing to the insufficient number of cases per mutation, previous studies have focused on the comparisons between wild-type and mutant p53, or between large categories of p53 mutants. To our knowledge, no existing empirical research addresses the effects of individual p53 mutations on human cancers.

In this study, we combine large-scale cancer genomic data to characterize the effects of each hotspot p53 mutation on human cancer prognosis. The potential pathways that may mediate drug resistance and cancer mortality were also analyzed to provide insight into the unequal mutant GOF effects in human cancers.

Results

Mutations on Arg282 and Arg248 residues associate with shorter patient survival. To assess the effects of different p53 mutations in cancer outcome, we firstly performed survival analysis on the MSKCC bladder cancer data set (JCO, 2013) in combination with The Cancer Genome Atlas (TCGA) colorectal cancer data set. Finally, the effects of p53 mutation and expression of CYP3A4 in response to FOLFIRI and cetuximab were analyzed using the indicated data sets.
Genome Atlas (TCGA) patient cohorts that had higher frequency of p53 mutation and adequate patient outcome data. These include breast invasive carcinoma, colorectal cancer, glioblastoma, lung squamous cell carcinoma and ovarian serous cystadenocarcinoma (analysis flow-chart shown in Figure 1d). These data sets include a total number of 2916 cases, and the mutation spectrum of TP53 gene was similar to that reported previously. The overall survival was compared between patient groups with different p53 status, namely nonsense (loss-of-function (LOF)) mutation group and hotspot (GOF) mutation group. To analyze if mutant p53 aggregation or abolished contact with DNA is associated with patient outcome, the patients were also classified according to the type of p53 mutations.

Kaplan–Meier survival analysis revealed that patients carrying p53 mutations on Arg248 and Arg282 residues had significantly shorter overall survival time than those carrying nonsense mutations (Figures 2a and b). Other frequently-occurring mutations at residues Y220, G245, R175 and R273 (Figures 2c and d) showed similar survival curves as nonsense mutations, and very few cases with the R249S mutation are available for further analysis. No difference in survival was found between structural, contact and nonsense mutations (Figure 2e). The frameshift, nonsense and missense mutations displayed comparable survival curves (Figure 2f), and even the comparison between wild-type and mutant p53 showed no significant difference in patient survival (Figure 2g). In the multivariate (Cox regression) analysis adjusting for cancer types, mutations on R282 and R248 residues showed ~ two-fold higher hazard ratios than that of the nonsense mutations (Figure 3a).

Validation of mutant effects using an independent data set. We tested the association between R248/R282 mutations and cancer patient survival using an independent data set extracted from published literatures (data in Supplementary Table 1), and found these mutations are indeed associated with shorter patient survival (Figure 3b). Multivariate survival analysis adjusting cancer types also confirmed this finding (Figure 3c). In fact, mice carrying humanized mutation on R248 (but not R175 or R273) were also found with significantly shorter survival time.

Figure 2 Univariate analysis on the effects of p53 mutations on cancer patient survival. Each hotspot mutation was compared with nonsense mutation using Kaplan–Meier survival analysis, and the P-values are indicated on the graph. Significantly shorter survival was found in patients baring p53 mutations on residues R282 (a) and R248 (b), but not on R175, R273 (c), Y220, and G245 (d). No significant difference was found between nonsense/structural/contact mutations (e), or between nonsense/frameshift/missense mutations (f). The wild-type and mutated p53 (including hotspots) showed no significant difference for overall patient survival (g).
suggesting that frequently-occurring p53 mutations display unequal GOF effects in both human and mouse. Although the structural/contact classifications seem to be uncorrelated to patient survival, all the mortality-associated mutations are located in p53’s binding surface to Bcl-XL (shown in Figure 1c), suggesting potential relevance of p53 mitochondrial apoptotic functions with cancer patient survival.

R248 and R282 mutations associate with drug metabolism enzymes. To probe the characteristic pathways associated with different p53-hotspot mutations, we selected the colorectal cancer (CRC) data set in TCGA as an example. The CRC cases contain more mortality-associated mutations (R248Q/W and R282W) and two intensively studied mutations (R175H and R273H), and thus were more suitable for further analysis. We performed Gene Set Enrichment Analysis (GSEA) to identify the specifically upregulated genes/pathways for each mutation as compared with nonsense mutations. When genes with top 1% enrichment scores were regarded as enriched genes (n = 177, listed in Supplementary Table 2), R248W and R282W shared 52 commonly enriched genes. However, such similarity was not found between other mutations (Figure 4a). When mutations were classified according to their gene enrichment profiles, R248W and R282W fell into the same subgroup (Figure 4b). The mortality-associated mutations were both enriched in gene sets linked to mitochondria outer membrane, cellular respiration and drug metabolism enzymes (Figures 4c–f). The R273H mutation shared serine–hydrolase pathway with R282W, but R175H did not show significant similarity with other mutations (Figure 4c).

Mortality-associated P53 mutations induce CYP3A4 upregulation. The GSEA analysis revealed that most of the drug metabolism enzymes in association with p53 R248/R282 mutations are responsible for the clearance of chemotherapeutic drugs. Among these, the most relevant one was cytochrome p450 (CYP3A4), which is the main metabolizing enzyme for FOLFIRI (folinic acid/fluorouracil/irinotecan) regimen, etoposide, rapamycin and other antineoplastic drugs (Supplementary Table 3). We tested the effects of mutant p53 on CYP3A4 by ectopic expression...
of p53 mutants R175H, R273H and R282W in the H1299 cells (p53-null). Immunofluorescence and Western blot studies suggested that p53 R248 and R282 mutations induced higher expression of CYP3A4 protein (Figures 5a–c, full gel image for Wb shown in Supplementary Figure 1 online). Consistently, real-time quantitative reverse transcription PCR (qRT-PCR) detected increased expression of CYP3A4 mRNA in H1299 cells expressing the mortality-associated mutants (Figure 5d). When p53 mutants were expressed in another p53-null cell line SaOS2, increased CYP3A4 mRNA and protein levels were also observed for R248 and R282 mutations (Supplementary Figure S2). Further, we tested the
association between p53 mutation and CYP3A4 expression in the Cancer Cell Line Encyclopedia (CCLE) data set, which contains drug response data for 639 human cancer cell lines with known genotypes. As expected, cancer cells bearing mortality-associated p53 mutations (R248, R282) displayed significantly higher level of CYP3A4 when compared with neutral GOF mutations (R175, R273) and nonsense LOF mutations (R175, R273) using Mann–Whitney test (P-value indicated). The CYP3A4 expression level decreased after mutant p53 was knocked down using specific siRNAs against p53 (data represent means ± S.D., P < 0.05, two-sided t-test). The CYP3A4 expression level also decreased (data represent means ± S.D., P < 0.05, two-sided t-test).

Figure 5 Effects of p53 mutations on CYP3A4 expression. (a) Different p53 mutations were ectopically expressed in human H1299 cells (p53-null), and immunofluorescence was used to detect p53 (in red) and CYP3A4 (green). Cell nuclei were stained by DAPI in blue. Scale bars indicate 10 μm. (b) Statistical analysis of CYP3A4 fluorescence in cells expressing different p53 mutants. Data represent means ± S.D. of four biological repeats. (c) Western Blot showing the level of CYP3A4 in H1299 cells expressing the indicated p53 mutants. Higher level of CYP3A4 protein expression was found in cells expressing p53 R248W and R282W mutations. (d) Quantitative RT-PCR detection of CYP3A4 mRNA expression in cells stably expressing different p53 mutations as indicated (data represent means ± S.D., P < 0.05, two-sided t-test). (e) CYP3A4 expression in cancer cells bearing different p53 mutations. The expression level of CYP3A4 was extracted using microarray data of Cancer Cell line Encyclopedia (CCLE) database. Then, the level of CYP3A4 was compared between cell bearing p53 mortality-associated mutations (on R248 or R282) or other mutations (on R175 R273) using Mann–Whitney test (P-value indicated). (f) The mRNA level of CYP3A4 in SW837 cells (bearing p53 mutation R248W) was measured by qRT-PCR. The CYP3A4 expression level decreased after mutant p53 was knocked down using specific siRNAs against p53 (data represent means ± S.D., P < 0.05, two-sided t-test). (g) The CYP3A4 expression level in HuP-T3 cells (carrying p53 mutation R282W) was determined by qRT-PCR. After p53 was suppressed by specific siRNAs, the CYP3A4 expression level also decreased (data represent means ± S.D., P < 0.05, two-sided t-test).

Cyp3a4 may mediate chemoresistance of P53 mortality mutations. To clarify the relationship between CYP3A4 expression and resistance to FOLFIRI and cetuximab, we compared the expression levels of CYP3A4 in CRCs displaying different therapeutic responses (GEO data sets GSE13294 and GSE5851). In this context, higher level of CYP3A4 also significantly decreased (Figures 5f and g). These results collectively demonstrate that mutant p53 R248W and R282W could induce the expression of CYP3A4 enzyme.
CYP3A4 was found in nonresponse groups than in response groups treated by both drugs (Figures 6a and b). These results suggest that higher CYP3A4 level may contribute to the chemoresistance associated with p53 mortality mutations. To test the effects of p53 GOF mutations on cell response to chemotherapy, 20μM etoposide was added to H1299 cells stably expressing p53 mutations (R175H, R273H, R248W or R282W) or the control vector. As a result, cells bearing R248W and R282W mutations showed higher viability than the control group after incubation with etoposide for 48 h (Figure 6c). In support of these findings, cancer cells bearing mortality-associated mutations displayed increased inhibitory concentration (IC50) for etoposide, rapamycin, elesclomol, MK-2206 and NVP-BEZ235 (Figure 6d, Supplementary Table 5). Notably, all of the above drugs are substrates of CYP3A4, supporting our notion that CYP3A4 may have important roles in p53 mortality mutation-associated drug resistance (illustrated in Figure 6e).

**Discussion**

Accumulating evidences suggest that p53 mutants display diverse oncogenic functions, and therefore should be considered as different proteins. However, most existing evidences are based on cellular and transgenic animal models, and strong support from human cancers is awaited. By characterizing the effects of hotspot p53 mutations on cancer patient survival, we present the first evidence that p53 missense mutations display different patterns and strengths of GOF activity in human cancers. Hotspot p53 mutations on two sites, namely R282 and R248 are associated with significantly shorter patient survival when...
compared with nonsense mutations. Previous studies have demonstrated that mice carrying mutations equivalent to human R175H and R273H showed no difference in survival time as compared with null mutation, although a broader tumor spectrum was observed. A recent study using humanized p53 mutant knock-in mice provided evidences that the R248Q mutation could significantly decrease survival time, but this effect was not found for G245S mutation. Our findings in human cancers are consistent with these results obtained from transgenic mouse models. To our knowledge, the GOF activity of R282W has not been characterized previously in mice.

Although each mutant p53 displayed unique set of enriched genes, a higher degree of similarity has been found between mortality-associated mutations. Importantly, we found that the p53 mutations on R248 and R282 could induce the expression of CYP3A4, which is one of the most important enzymes involved in the metabolism of chemotherapeutic drugs in the human body. Consistently, cancer cells bearing p53 R248/R282 mutations displayed resistance to multiple CYP3A4-metabolized antineuplastic drugs. These findings provide mechanistic insight into the mortality effect of a subset of p53 mutants.

In summary, we provide the first evidence for the unequal GOF effects of p53-hotspot mutations in human cancers, and probed the potential mechanisms by which R248/R282 mutations may lead to chemoresistance and mortality. These findings add an important piece of evidence to GOF effects of the p53 mutant, and suggest that p53 mutations have unequal prognostic significance in human cancers.

Materials and Methods

Plasmids constructions. The pcDNA3-HA-p53 expression construct was constructed by inserting PCR-amplified cdNA sequences into pcDNA3 vector (Invitrogen, Carlsbad, CA, USA). All p53 mutations (R175H, R273H and R282W) were generated by site-directed mutagenesis PCR reaction using platinum PWO SuperYield DNA polymerase (Roche, Basel, Switzerland) according to the product manual. All plasmids were sequenced to confirm if the designed mutation is present, without any other unwanted mutation.

Cell culture and transfection. The human H1299 cells were maintained in Dmem medium (Gibco, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (Invitrogen) and cultured in a humidified incubator at 37 °C under 5% CO2. For transfection, cells were seeded into normal growth medium at 50% confluence in six-well plate 24 h before transfection, and then transfected using FuGENE HD (Promega, Fitchburg, WI, USA) according to the product manual. Briefly, the transfaction complex was made by 1 μg plasmid, 3 μl FuGENE HD and 100 μl medium. Six hours after the complex was added to the cells, normal culture media was used to culture cells for additional 48 h, followed by gene expression analysis. siRNA for p53 has the sequence of 5′-GACUCCAGU GGUAAUCUC-3′. Stable clones expressing different p53 mutations were selected with 600 μg/ml G418 for 12 weeks, and the expression of p53 was confirmed by immunofluorescence.

Quantitative real-time PCR analysis. Total RNA was extracted from H1299 cells using TRIzol reagent (Invitrogen). Reverse transcription was done using the RevertAid M-MuLV reverse transcriptase enzyme (Fermentas, Glen Burnie, MD, USA) according to the manufacturer’s protocol. Quantitative PCR was performed using an ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA) using SYBR Premix Ex Taq II (Takara). Quantitative PCR was performed using an ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA) using SYBR Premix Ex Taq II (Takara). Quantitative PCR was performed using an ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA) using SYBR Premix Ex Taq II (Takara). Quantitative PCR was performed using an ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA) using SYBR Premix Ex Taq II (Takara).
mutation than those with nonsense mutations, and the set was termed ‘enriched’ in the specified hotspot mutation.

Clustering analysis. Different p53 hot spot mutations (R175H, Y220C, G245S, R248W, R248Q, R273H and R282W) were clustered according to their enrichment scores for each gene (based on GSEA analysis). The hierarchical clustering was carried out using the Cluster program (Pearson correlation, average linkage) and visualized by TreeView (EisenLab, Lawrence Berkeley National Laboratory, Berkeley, CA, USA).

Conflict of Interest
The authors declare no conflict of interest.

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Author contributions
J Xu–study concept and design, acquisition of data wrote the paper; J Wang–acquisition of data, wrote the paper; Y Hu, J Qian, X Xu, H Chen, W Zou–performed experiments and analyzed data; J–Y Fang–study design, wrote the paper.

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