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News and Commentary

Polymorphic variants in the p53 pathway

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Introduction

Of all the tumor suppressor gene pathways that play a role in tumor development, few are as important as the p53 pathway. Commonly regarded as the most frequently mutated gene in human cancer, p53 is inactivated by mutation in over 50% of human tumors. Given the importance of the p53 pathway in suppressing tumor development, it is somewhat surprising to discover the existence of polymorphisms in genes in this pathway that impair its function. These include two polymorphisms in the *TP53* gene itself, as well as one in the promoter of *MDM2*. Also evident are polymorphisms in p53 consensus elements in the promoters of target genes. The functional consequences of these polymorphisms, and their influence on cancer risk, progression, and therapy, are discussed here. Potential reasons why decreased function of this pathway might be selected for in certain populations are discussed at the end of this chapter.

Polymorphisms in *TP53:* the codon 72 polymorphism

The cDNA for p53 was first cloned in 1984;[1–3] approximately 2 years later, a mobility shift in p53 protein was identified as a sequence polymorphism at amino acid 72, changing proline to arginine.[4–6] Unfortunately, at the time of its discovery, researchers were inadvertently studying a tumor-derived mutant form of p53, with the belief that *TRP53* was an oncogene. Therefore, the only functional analysis performed for these polymorphic variants was for their transforming potential, which did not differ, so the polymorphism was deemed functionally insignificant. Only in 1994, when analyzing the allele frequencies of the proline 72 and arginine 72 variants (P72 and R72, respectively) in human populations did Beckman and co-workers note statistically significant differences in allele frequency between different ethnic groups. For example, the P72 allele frequency is approximately 60% in African Americans, but only 30–35% in Caucasian Americans. Interestingly, Beckman noted that the P72 allele frequency increases in a linear manner in multiple populations as they near the equator.[7] This led him to hypothesize that the codon 72 polymorphism might have an impact on p53 function, and that the high exposure to UV light in populations near the equator led to selection for the P72 allele.

In 1999 Banks and co-workers performed the first comparison of the biological activities between P72 and R72 proteins, using transient transfection assays to measure p53 activity. The authors found no differences in the DNA binding or transcriptional properties of these two proteins, but they did find that the R72 form of p53 was a better suppressor of cellular transformation; this function of p53 is commonly believed to rely on its ability to induce programmed cell death, or apoptosis.[8] This group also found that the kinetics of apoptosis induction for R72 were approximately five times faster than P72. A subsequent paper by this group indicated that the R72 variant of p53 was more susceptible to degradation by the E6 protein of transforming variants of human papillomavirus (HPV) types 16 and 18. This study included a small-scale population analysis of women in Italy, revealing that the increased susceptibility to degradation by E6 was mirrored by an increased incidence of cervical cancer in HPV-infected women who were homozygous for the R72 variant.[9] This correlation between codon 72 variants and susceptibility to cervical cancer has failed to hold up in other population studies, although it should be noted that there are many different subtypes of HPV, and differences in the subtype of virus studied may account for the differences in findings between different groups.

The first comparison of the biological activity of endogenous P72 and R72 proteins was performed by Franceschi in 2002; in that study, blood leukocytes homozygous for R72 were found to undergo significantly increased apoptosis in response to the cytotoxic drug cytosine arabinoside, compared to P72.[10] Our group later group performed a comparison of cell lines containing inducible versions of p53, as well as human tumor cell lines homozygous for each variant. These analyses revealed a consistent five- to 15-fold increased ability of the R72 form of p53 to induce apoptosis.[11] Like the Banks study, a broad-scale analysis of the DNA binding and transcriptional properties of these two variants failed to reveal the basis for the increased apoptotic potential of R72. Further analysis, however, revealed that the R72 variant demonstrated greatly increased trafficking to mitochondria, where Moll and co-workers had reported in 2000 that p53 had direct ability to induce cytochrome *c* release and programmed cell death.[12] Indirect immunoflourescence provided the first clues as to mechanistic basis for this increased mitochondrial trafficking of R72. Specifically, cells with the R72 protein had extensive cytosolic immunostaining for p53, whereas cells with P72 had an almost exclusively nuclear stain. It was known at the time that nuclear export of p53 was controlled by the ubiquitin ligase Mdm2,[13,14] and further studies revealed that the binding affinity for Mdm2 was three- to five-fold greater for R72 protein than P72, thereby leading to enhanced

nuclear export of this variant.[11] In sum, while low levels of P72 protein can be found trafficking to mitochondria, significantly more R72 protein can be found associated with this organelle. The next challenge for researchers was the identification of the mechanism whereby mitochondrial p53 induced programmed cell death.

Using mass spectrometry analysis of immuno-complexes isolated from highly purified mitochondria, our group, along with the group of George, identified Bak as a mitochondrial p53-interacting protein. We showed that p53 binds to Bak directly, and that the Bak-binding domain of p53 overlaps with a conserved region of the DNA-binding domain. Using highly-purified mitochondria and recombinant p53, we showed that p53 can directly oligomerize Bak, leading to cytochrome *c* release.[15] Youle and co-workers previously reported that Bcl2 family members like Bak can undergo artificial detergent-induced conformational changes in cells lysed in nonionic detergents, leading to artificial protein–protein interactions.[16] Therefore, it is important to note that our group showed that the p53- Bak interaction exists even when cells are lysed in Chaps buffer, which does not induce these artificial conformational changes.[15,16] Interestingly, we found that recombinant P72 and R72 can bind to Bak identically, so the consequences of the codon 72 polymorphism appear to impact only nuclear export, and not Bak interaction. Indeed, we showed that direction of both forms of p53 to mitochondria, by fusing each variant to the mitochondrial leader peptide from ornithine transcarbamylase, allowed both variants to traffic to mitochondria and induce cell death equivalently.[11] In related studies, two other groups reported that p53 interacts with (other) Bcl2 family members; Green and co-workers found that p53 could directly oligomerize Bax, and Moll and co-workers reported that p53 can interact with Bcl-xl.[17,18] While these groups did not check to see if these interactions are affected by this polymorphism, it can be surmised that the codon 72 polymorphism of p53 likely affects the overall transcription-independent mitochondrial pathway of cell death; an in-depth review of the molecular components of this pathway can be found in an accompanying chapter of this edition, by Chipuk and Green (this issue).

Impact of the codon 72 polymorphism on cancer risk, progression, and treatment efficacy

A number of studies have attempted to determine if there is an association between codon 72 polymorphic variants of *TP53* and risk for particular cancer types. These studies have come to very varied conclusions, with some studies reporting increased risk associated with the P72 allele for certain cancer types[19–21] and others failing to reach such conclusions.[22–24] The reasons for these discrepancies are not clear, but it can be safely said that if such associations exist, they may not be particularly strong, or they be influenced by unknown variables that presently are not controlled for in such studies. In contrast, there are more consistent associations between the codon 72 polymorphism with cancer progression, age of onset, and overall survival. For example, a recent study analyzed 93 patients diagnosed with Hereditary Nonpolyposis Colorectal Cancer (HNPCC); these individuals had germline mutations in the mismatch repair genes *MLH1* or *MSH2*. This study found that individuals with at least one copy of the P72 allele had a median age of onset for disease that was 13 years earlier than individuals who were homozygous for the R72 allele.[25] Analyses by other groups have revealed an 11 year earlier age of onset in individuals with at least one P72 allele for oral cancer, and a 6 year earlier age of onset for squamous carcinoma of the head and neck.[26] In terms of the efficacy of therapy, Crook and co-workers found that the P72 form of p53 mediates a less efficient response to chemotherapy, both *in vitro* and *in vivo*. In particular, this group reported that in 43 patients with inoperable head and neck cancer whose tumors retained a wild-type *TP53* allele, individuals whose cancer retained an R72 allele had significantly enhanced chance for complete response, overall survival, and progression free survival ($P<0.04$, $P=0.02$ and $P=0.007$, respectively), compared to P72.[27] All of these trends may be explained by the increased apoptotic potential of the R72 variant, though this has yet to be formally tested.

Polymorphisms in *TP53*: the codon 47 polymorphism

A second coding region polymorphism exists in p53 at codon 47. Harris and co-workers first discovered this polymorphism, which changes proline to serine at amino acid 47, in 1996. In that study, the allele frequency was found to be $<5\%$ in African Americans, and not at all in Caucasians.[28] Our group found the S47 allele frequency to be approximately 1% in African Americans.[29] The S47 polymorphism resides next to serine 46, whose phosphorylation is a critical controlling event for p53-mediated apoptosis; notably, serine 46 is phosphorylated by proline-directed kinases like p38Mapk and Hipk2 (homeo-domain-interacting protein kinase 2), and such kinases require an adjacent proline to direct phosphorylation. Therefore, the S47 polymorphism would be predicted to impair phosphorylation on serine 46. In line with this, we found that S47 protein is a markedly poorer substrate for phosphorylation on serine 46, and has impaired ability to induce apoptosis.[29] We found that this polymorphism occurs in *cis* with P72 (and may be in linkage disequilibrium with this allele) and does not affect the transcription-independent mitochondrial pathway of cell death. Rather, in addition to decreased phosphorylation on serine 46, the S47 variant has impaired ability to transactivate the p53 target genes *p53AIP1* and *PUMA*.[29] The influence of this polymorphism on cancer risk, progression and efficacy of therapy awaits epidemiological studies; however, the low frequency of this polymorphism may prohibit such studies. Alternatively, the generation of mouse models for these p53 polymorphic variants could shed light on these issues.

A polymorphism in the noncoding region of *MDM2* alters its transactivation potential

Levine and co-workers first noted significant variations in the p53 response in lymphocytes isolated from 50 healthy

volunteers. This finding prompted them to analyze the *MDM2* gene for sequence polymorphisms that might contribute to this inter-individual variation in the p53 response. They discovered a functionally significant single nucleotide polymorphism (SNP) in the promoter for *MDM2*; this polymorphism occurs at nucleotide 309 of intron 1 of the *MDM2* gene, and changes a T to a G (the 'G' allele is denoted SNP309). The T allele frequency was reported to be 0.58, and G was 0.32, making this a common polymorphism. Notably, this change is predicted to create a higher-affinity binding site for the transcription factor Sp1, which is an important controller of *MDM2* mRNA levels. In line with this, this group demonstrated that the G allele bound with two- to four-fold enhanced affinity to purified Sp1 *in vitro*, as well as *in vivo* using chromatin immunoprecipitations in cells homozygous for the G allele. Additionally, this group noted a consistent correlation between cell lines homozygous for the G allele with increased steady state levels of Mdm2 protein.[30]

Functionally speaking, cell lines homozygous for the G allele of SNP309 were shown to have an attenuated p53 transcriptional and apoptotic response, due to a decreased ability of p53 to stabilize following DNA damage. This group then analyzed individuals from families with Li–Fraumeni syndrome, who have one nonfunctional copy of the p53 gene, and therefore already have an attenuated p53 response. This group hypothesized that the *MDM2* SNP might further attenuate the p53 pathway in these individuals, and therefore affect cancer incidence in these families. Analysis of 88 individuals from Li Fraumeni families containing germline mutations in one allele of p53 revealed that individuals who carried the G allele of SNP309, in either the heterozygous or homozygous state, showed a significantly earlier age of onset for all tumor types (a minimum of 9 years earlier). Additionally, individuals homozygous for the G allele of SNP309 also had an increased occurrence of independent subsequent cancers.[30]

In addition to impaired stabilization of p53 associated with the G allele of SNP309, Bargonetti and co-workers recently reported that cell lines homozygous for this allele have an impaired transcriptional response of p53, independent of the attenuated stabilization. Specifically, chromatin immunoprecipitation analysis of p53 target genes indicated that in cells homozygous for the G allele of SNP309, p53 could be found bound to the promoters of p53-target genes following treatment with chemotherapeutic agents. This p53 protein was found to be appropriately phosphorylated in serine 15, indicating the upstream signaling pathway from DNA damage was intact. However, p53 binding to these response elements was not accompanied by transactivation of such genes because *MDM2* was found complexed as well;[31] Mdm2 is known to inhibit p53 transactivation by concealing the transactivation domain of this protein.[32] Downregulation of *MDM2* in these cells, using siRNA, restored the p53-responsiveness of p53 target genes in these cells.[31] The take home message from such studies is that the increased expression of Mdm2 caused by SNP309 impairs the p53 pathway.

Several groups have analyzed the impact of SNP309 in MDM2 on cancer risk and progression. Several groups report that the G allele is associated with attenuation of the p53 pathway and enhanced early onset of, and increased risk for, tumorigenesis.[33–36] In contrast, several other groups have found no association between SNP309 and cancer risk or age of onset.[37–40] It is possible that the impact of this polymorphism may differ for different tumor types, or may depend upon the initiating molecular event that drives tumor cell development. Along these lines, two independent reports have shown that germline p53 mutation carriers who possess the G-allele of SNP309 were diagnosed with cancer on average 7–10 years earlier than those who were homozygous for the T allele.[30,33] There have also been two reports that address a possible interaction of the SNP309 and the codon 72 polymorphism in the p53 gene.[33,36]

Polymorphic p53 binding sites in the promoters of p53 response genes

Heterogeneity of the p53 response in individuals may also come from differences in p53 response elements in target genes. Resnick and co-workers first noted that in the p53 response elements isolated from 26 different p53 target genes, there were 1000-fold differences in transactivation potential by p53.[41] In this study, the central sequence element in between p53 monomer binding sites (5′ CATG 3′) was determined to markedly affect p53 transactivation capacity. With the knowledge that apparently minor alterations in p53 consensus elements could dramatically impact transactivation potential by p53, Resnick and co-workers took this study one step further, and searched for genetic variation in promoter response elements for p53. Using a customized bioinformatics assay, combined with eucaryotic functional assays, this group identified a minimum of six *bona fide* p53-response genes with polymorphisms in their response elements that significantly impacted their transactivation potential by p53.

Functional p53 binding sites consist of the consensus element RRRCWWGYYY-N-RRRCWWGYYY, where R is purine, Y is pyrimidine, W is A or T, and N is 0–13 bases.[42] In general, however, Resnick and others have noted the following: (i) most functional response elements have a spacer length of 0–1 nucleotide; (ii) most sites have fewer than four total mismatches from the consensus, and no more than three mismatches in one 10 nucleotide half site; (iii) changes in the conserved C or G can dramatically affect transactivation; (iv) within the WW motif, AT provides the strongest transactivation; (v) mismatches in the R and Y bases have a greater negative impact on transactivation the closer they are to the central CWWG motif; (vi) most p53 response elements fall within a few thousand nucleotides of transcriptional start sites. Using these rules, this group identified approximately 40 SNPs that were predicted to occur in functional p53 response elements, and have a significant impact on transactivation by p53. Interestingly, response elements mapping close to three genes, *DCC, TLR8*, and *ADAR2* (Deleted in colorectal carcinoma, Toll-like receptor 8 and Adenosine deaminase acting on RNA) had common polymorphisms (frequency >0.25) that dramatically altered their p53 inducibility.[43] The contribution of these polymorphic variants to the known differences in p53

response, or to differences in cancer risk and prognosis, remains to be addressed.

Conclusions

Table 1 lists the polymorphisms in the p53 pathway discussed here, as well as other functionally significant polymorphisms in this pathway. With p53 at the helm of this major tumor suppressive pathway, at least two relevant questions emerge: first, what is the impact of these polymorphisms on cancer risk and prognosis? This question is only just now being addressed appropriately, with researchers controlling not only for tumor type, but also for tumor initiating event (e.g., *MSH2* or *MLH1* mutation in HNPCC). The second, perhaps even more fundamentally intriguing question, is why would polymorphisms that inactivate such a critical tumor suppressive pathway exist in human populations? What possible selective advantage could this offer? While it remains entirely possible that there are no selective advantages keeping these variants in the population, there are some intriguing possibilities. One possibility is provided by the studies of Van Heemst and coworkers, indicating that the codon 72 polymorphism (and perhaps other polymorphisms in this pathway as well) has an influence on longevity. This group performed a prospective study of inhabitants of Leiden in the Netherlands; 1226 individuals over the age of 85 were genotyped for the codon 72 polymorphism, and followed for specific causes of death for many years. As might be predicted, proportional cancer mortality was increased for homozygous P72 individuals compared to homozygous R72 (29 *versus* 14%, respectively); this significant difference is consistent with the increased apoptotic potential of R72. Interestingly, however, this same study indicated that homozygous P72 individuals had a 1.41-fold relative survival increase over their homozygous R72 counterparts, with deaths from noncancer-related events such as general exhaustion and frailty occurring in 21% homozygous R72, but only 6% homozygous P72.[44] Indeed, studies in mouse on hypermorphic *TP53* indicate that a more active form of p53 decreases cancer risk, but also shortens lifespan.[45] So the enhanced lifespan associated with the lesser active P72 allele may outweigh the deleterious effects of cancer susceptibility, and select for this allele in the population.

An alternative hypothesis for the selection for an impaired p53 pathway in humans is also plausible. Crook and coworkers have detected a relationship between the p53 codon 72 polymorphism and skin phototype.[46] Specifically, individuals with R72 alleles are significantly more susceptible to sunburn, while those with P72 alleles tend to be darker skinned, and more readily tan in response to solar radiation ($P = 0.0001$ for trend). These trends would suggest that the P72 allele would be selected for near the equator, as the increase in melanized skin afforded by the P72 allele would protect individuals from the photolysis of folate that occurs with high UV absorption; folates are required for normal development of the embryonic neural tube. In contrast, away from the equator the R72 allele would be selected for, as it would allow for the increased UV absorption required for fixation of vitamin D, which is essential for normal growth, calcium absorption, and skeletal development. Indeed, deficiency in vitamin D can cause death, and individuals with highly melanized skin (such as might accompany the P72 allele) traditionally under-fix vitamin D, because darker skin

Table 1 Functionally significant polymorphisms in the p53 pathway

Gene	Polymorphism	Functional consequence	Reference(s)
TP53	Codon 72 CCC→CGC Pro→Arg	R72 induces apoptosis better; traffics to mitochondria better.	Thomas *et al.*[8] Bonafe *et al.*[10] Dumont *et al.*[11]
TP53	Codon 47 CCG→TCG Pro→Ser	S47 has decreased phosphorylation on serine 46, decreased apoptosis	Li *et al.*[29]
MDM2	SNP309 Nucleotide 309, first intron T→G	G allele has increased binding by Sp1; leads to increased expression of MDM2	Bond *et al.*[30]
CDKN1A	Codon 31 AGC→AGA Ser→Arg	May lead to decreased mRNA for p21; may increase risk for certain cancers	Su *et al.*[47]
CDKN1A	Codon 149 GAT→GGT Asp→Gly	Occurs in PCNA binding domain; may increase risk for certain cancers	Bahl *et al.*[48] Ralhan *et al.*[49]
PIG3	Pentanucleotide microsatellite repeat +442→+517	Increased repeat number leads to increased p53 inducibility	Contente *et al.*[50]

inhibits the absorption of UV light needed to maximize synthesis of previtamin D3. Certainly, it is easier to rationalize these polymorphic variants being selected for to accommodate increased vitamin D fixation and inhibition of folate photolysis, than increased longevity or altered cancer risk, whose selection would occur after child-bearing years. Whether or not similar associations between skin phototype and polymorphisms in other genes in the p53 pathway, such as *MDM2* or *CDKN1A*, exist remains an attractive hypothesis that awaits testing.

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