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Advances in Brief

Effects of *p51/p63* Missense Mutations on Transcriptional Activities of *p53* Downstream Gene Promoters[1]

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Abstract

The *p51/p63* gene is a homologue of *p53*, the product of which acts as a transcriptional activator by binding to p53-responsive elements in the promoter regions of several p53 downstream genes. Recently, we identified four distinct mutations in the *p51/p63* gene after screening >200 human tumors and cell lines. Because all of the detected *p51/p63* mutations were missense mutations, the pathogenic effect of these mutations is difficult to determine without performing a functional analysis. In this study, we examined the transcriptional activity of tumor-derived *p51/p63* missense mutations using a yeast-based assay and compared the data with that of artificial *p51/p63* missense mutations at residues corresponding to the positions and substituted residues of *p53* mutation "hotspots." Although most of the *p51/p63* missense mutations at the *p53* hotspot residues were unable to transactivate the promoters used in this study, the tumor-derived *p51/p63* missense mutations retained their ability to transactivate the *MDM2* and/or the *BAX* promoter but not the *p21/WAF1* promoter. These results suggest that the p51/p63 mutation might be involved in an unknown tumor suppression pathway distinct from that of p53.

Introduction

The tumor suppressor protein p53 acts as a transcriptional activator. In the presence of genotoxic stresses, p53 is activated by the phosphorylation or acetylation of a subset of residues (1–3), forms a homo-tetramer, and binds to specific DNA sequences (4) within the promoter regions of target genes located downstream. These target include *p21/WAF1, MDM2, BAX, 14–3-3σ*, and others and are mainly involved in the arrest of the cell cycle or the induction of apoptosis (5–11). *p53* mutations are observed frequently in various types of human cancers (12) and are mostly missense mutations.[4] In general, the *p53* missense mutations result in the inactivation of the protein, preventing it from binding to the p53-responsive elements. However, the degree of inactivation varies for each p53-responsive promoter region and for each missense mutation (13, 14). For example, some p53 mutants retain their ability to activate the *p21/WAF1* promoter but are unable to activate the *BAX* promoter. These differences may allow some cancer cells to become resistant to certain anticancer drugs (15).

Recently, the *p51/p63* gene was isolated (16, 17). This gene is a member of the *p53* structurally related gene family (18). Although the *p51/p63* gene can encode multiple isotypes through several alternative splicing patterns, the predicted amino acid sequences in the p51 central region (which corresponds to the DNA binding domain in p53) share a 60% identity with those of p53. The similarity in structures between p53 and p51/p63 suggests that p51/p63 also acts as a transcriptional activator. In fact, one of the splicing variants of p51/p63 (p51A/p63γ) has the ability to up-regulate promoters, including p53-responsive elements, in both yeast and mammalian cells (16, 17, 19). These results strongly suggest that not only the structure but also the function of these two related proteins is highly conserved.

In contrast to *p53*, only a few *p51/p63* missense mutations have been observed. To date, a screening of human tumor cells and human tumor derived cell lines has detected only seven missense mutations within the *p51/p63* coding sequence (16, 20–22). Because the pathogenic effect of these missense mutations cannot be elucidated without a functional assay, we recently examined two of these missense mutations for sequence specific transactivation. Missense mutation p51A148P inactivated the ability of p51/p63 to transactivate the *p21/WAF1* promoter, but missense mutation p51Q31H did not (20). However, whether these two mutations and the other missense mutations can act as functional mutations for the *p21/WAF1* promoter and other p53 up-regulating gene promoters remains unclear. Furthermore, determining whether the function of p51/p63 can be inactivated by a missense mutation may provide a clue to the reason why so few p51/p63 mutations have been found in tumors.

In this study, we examined the functional effects of four tumor-derived *p51/p63* missense mutations and artificial *p51/p63* missense mutations that mimic p53 "hotspot" mutations. The ability of the missense mutations to inactivate transcriptional activity was investigated using a transcription assay in yeast.

Materials and Methods

Yeast Strain and Plasmids. The yeast strain used in this study was YSIS (*MATa, ura3–1, ade2–1, trp1–1, his3–11, leu2–3, 112, can1–100, pep4::URA3*). All p51/p63 and p53 mutation expression plasmids were constructed using mega-primer methods (23) and, except for the specific mutations, were identical to pLSC53A and pCIP51–2, respectively (19). All plasmids were sequenced to confirm that the appropriate mutation had been incorporated and that no additional mutations were present. The wild-type p51A/p63γ and p53 expression vectors (pCIP51–2 and pLSC53A) and the GFP[5] reporter plasmids containing the *p21/WAF1* (pAS03G), *MDM2* (pAS05G), *BAX* (pAS07G), and *14-3-3σ* (pAS09G) promoter regions were described in a previous report (19).

Assay for Transcriptional Activity in Yeast. The assay for detecting transcriptional activity in the yeast was described previously (19). Each expression vector and reporter plasmid were cotransformed into YSIS and grown at 30°C on a solid synthetic complete medium lacking leucine and tryptophan (SC-leu-trp). The resulting colonies were assayed for GFP expression using a fluorescence microscope equipped with a GFP Plus filter (Fluoroskan Ascent FL; Dainippon, Tokyo, Japan). The intensity of the GFP fluorescence was

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[5] The abbreviations used are: GFP, green fluorescent protein; p51A148P, substitution of an alanine to a proline at codon 148 of *p51/p63*.

Table 1 *Histological type and the status of p51 and p53 of the cell p51 missense mutation derived*

p51 missense mutation (nucleotide change)	Origin (name)	Histological type (cancer type)	Wild-type p51 expression	p53 status	Reported p53 mutation at same residue	Ref.
Q31H (CAG to CAC)	Tumor (C17)	Adenocarcinoma (lung cancer)	Detected	mut(−)	none(not conserved residue)	(20)
Q31H (CAG to CAC)	Tumor (C47)	Adenocarcinoma (lung cancer)	Detected	Not determined	none(not conserved residue)	(20)
S145L (TCG to TTG)	Cell line (HO-1-u-1)	Squamous cell carcinoma (oral floor plate)	Not detected	mut(+)[a]	p53S116P, p53S116C[b]	(16)
A148P (GCC to CCC)	Tumor (C8)	Squamous cell carcinoma (lung cancer)	Not detected	Express only mutant allele mut(+)	p53A119R, p53A119T[b]	(20)
Q165L (CAA to CTA)	Cell line (SKG-IIIa)	Squamous cell carcinoma (cervical cancer)	Not detected	Express only mutant allele (HPV+) mut(−)[a]	p53Q136R, p53Q136E, p53Q136K[b]	(16)

[a] Jia *et al.* (28).
[b] Internet address: http://perso.curie.fr/Thierry.Soussi/p53_databaseWh.htm.

analyzed, using a fluoroscanmeter, and living yeast cells were grown at 37°C on a 96-well microtiter plate containing a SC-leu-trp medium (19).

Results and Discussion

Eleven low-copy centromeric vectors, each expressing the entire p51A/p63γ region plus a specific missense mutation, were constructed (see "Materials and Methods"). Four of the missense mutations were derived from human tumors: p51Q31H and p51A148P originated from human lung cancer tumors (20), and p51S145L and p51Q165L originated from two different human tumor cell lines (Ref. 16; Table 1). The remaining seven missense mutations, p51R204H, p51R204L, p51G276V, p51R279Q, p51R280S, p51R304H, and p51R313W, consisted of amino acid substitutions designed to mimic p53 hotspot mutations p53R175H, p53R175L, p53G245V, p53R248Q, p53R249S, p53R273H, and p53R282W, respectively (Fig. 1).

To examine the effects of the tumor-derived *p51/p63* missense mutations on the ability to transactivate p53 downstream promoters, each mutant p51/p63 expression vector as well as a wild-type p51/p63 and a wild-type p53 expression vector were cotransformed with a series of p53-resposive GFP reporter plasmids (containing the p53-responsive elements of the *p21/WAF1*, *MDM2*, *BAX*, or *14-3-3σ* promoter fragments) into a yeast haploid strain (YSIS). The GFP fluorescent intensity of the resulting colonies was then quantitatively analyzed at 37°C. As shown in Fig. 2, the wild-type p51/p63 vector activated the *p21/WAF1* reporter less effectively (Fig. 2*A*) and the *BAX* reporter more effectively than the wild-type p53 vector (Fig. 2*C*). Both the wild-type p51/p63 vector and the wild-type p53 vector activated the *MDM2* reporter to the same degree (Fig. 2*B*). The wild-type p51/p63 vector was unable to transactivate the *14-3-3σ* reporter, although the wild-type p53 vector could (data not shown). These results are consistent with our previous data (19). Among the

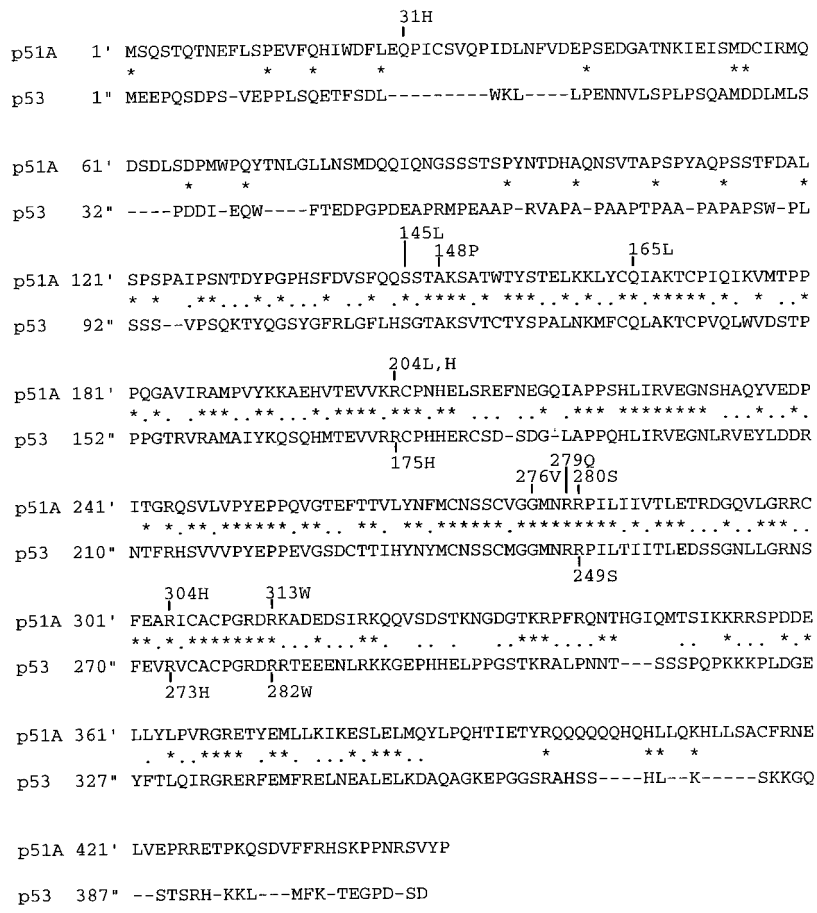

Fig. 1. Missense mutations in p51A/p63γ and p53. The specific sites and substituted residues are shown in the amino acid sequences of p51A/p63γ and p53. ★, an amino acid residue that is conserved between p51A/p63γ and p53; ., a similar amino acid residue between the two proteins.

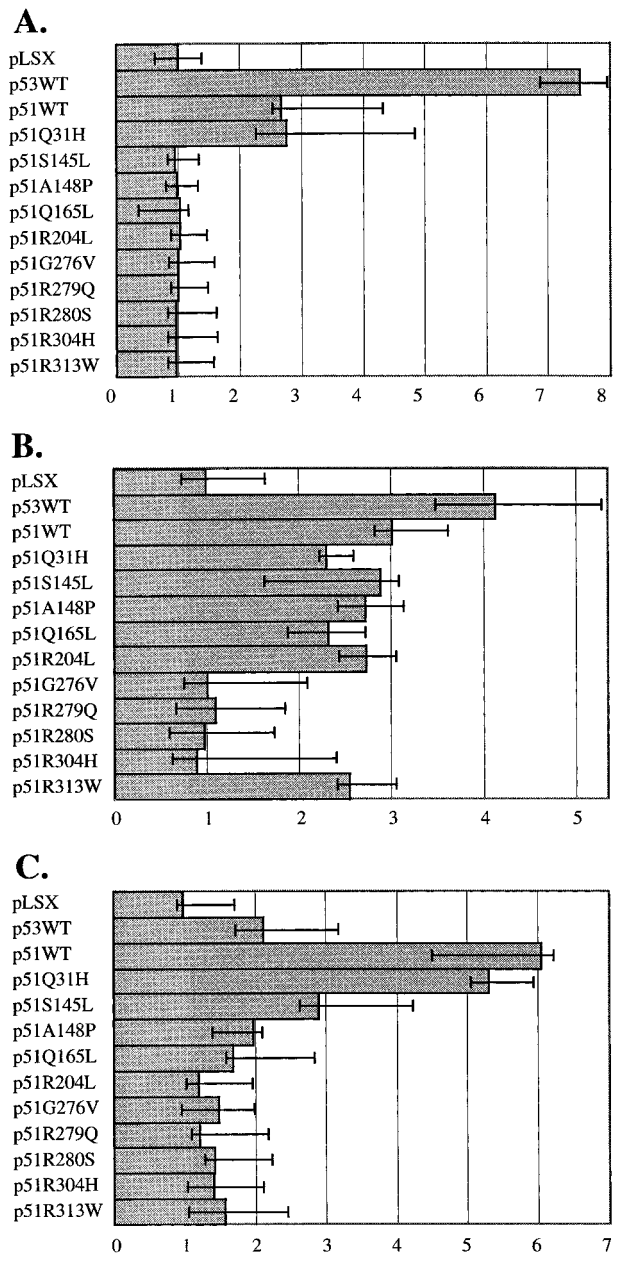

Fig. 2. Transcriptional activation activities of wild-type and mutant p51A/p63γ proteins in p53 target genes. *A, WAF1; B, MDM2*; *C, BAX* promoters. *X axis,* the green fluorescent intensity relative to a null expression vector (pLSX1). *Bars,* one SE of three values.

tumor-derived mutations, p51Q31H retained the ability to transactivate the *p21/WAF1*, *MDM2*, and *BAX* promoters at a level equivalent to that of the wild-type p51/p63 vector (Fig. 2). This observation is consistent with our previous data using a *HIS3* reporter construct that contained the *p21/WAF1* promoter (20) and suggests that this mutation is functionally silent. This finding is not surprising, because the transactivation domain containing the residue at codon 31 is not conserved between p51/p63 and p53 (Fig. 1) and because the inactivation of the transactivation domain by a single amino acid substitution seems to be difficult in the case of p53 (24). To date, no identical alterations have been found in more than 90 alleles derived from normal tissues (20), and no other biological functions of p51/p63 are known that could help to clarify the p51/p63 mutation. Therefore, the possibility that the p51Q31H mutation is either a rare polymorphism or a pathogenic mutation affecting an unknown function of p51/p63 cannot be excluded. The remaining tumor-derived mutations (p51S145L, p51A148P, and p51Q165L) were unable to transactivate the *p21/WAF1* reporter (Fig. 2*A*) but retained their ability to partially transactivate the *MDM2* and the *BAX* promoter (Fig. 2, *B* and *C*). These results indicate that each of the three tumor-derived missense mutations in the *p51/p63* gene affects, at least partly, the transactivation function of p51/p63 in a manner similar to that of p53. Interestingly, the backgrounds of the three mutations also share several similar genetic features. For example: (*a*) each mutation is located within the NH_2-terminal boundary region of the DNA binding domain; (*b*) each residue is highly conserved between p51/p63 and p53 (Fig. 1), and two or three distinct types of *p53* missense mutations have been reported (Table 1); (*c*) only the mutant transcripts were expressed in the tumors; (*d*) the p53 status of the original tumor or cell line was defective because of a mutation within the *p53* gene or the presence of a human papillomavirus (Table 1); and finally (*e*) each tumor or cell line was derived from squamous epithelium (squamous cell carcinoma), which requires p51/p63 during development in mice (25, 26). Although this information is insufficient to prove that the *p51/p63* mutations are involved in tumorigenesis, we speculate that the *p51/p63* mutations might play a role in the pathogenesis of some types of tumors.

Our initial screening for *p51/p63* mutations in more than 200 tumor and cell lines revealed only four distinct missense mutations in five cases (Table 1). We demonstrated previously that p51/p63 shares its downstream signals at least in part with p53 (19), although the upstream signals in the p51/p63 pathway are still unclear. From these observations, one may speculate that the biological function(s) of p51/p63 is distinct from that of p53, which serves as a "guardian" against genotoxic stress in cells, and that p51/p63 probably does not play a major role in tumor suppression, unlike p53. Alternatively, a single amino acid substitution in p51/p63 may be insufficient to inactivate the protein structure of the DNA binding domain. If this is true, the *p51/p63* gene would not be a sensitive target for the inactivation of the p51/p63 pathway via mutation. To examine the later possibility, the effects of the *p51/p63* missense mutations at residues corresponding to the positions of *p53* mutation hotspots (p51R204L, p51G276V, p51R279Q, p51R280S, p51R304H, and p51R313W) were examined for their ability to transactivate the p53-responsive promoters (Fig. 2). The transcriptional activities of the p53 hotspot mutations (p53R175H, p53R249S, p53R273H, and p53R282W) were also examined and compared with the data for the mutations designed to mimic them (p51R204H, p51R280S, p51R304H, and p51R313W, respectively; Fig. 3). Both the *p51/p63* mutations and the *p53* mutations inactivated the transcriptional activity of all of the p53-responsive reporters that were examined except for p51R204L and p51R313W, which retained their ability to activate the *MDM2* reporter. These results indicate that the *p51/p63* gene is a sensitive target for missense mutations within the DNA binding domain in a manner that is similar to that of the p53 inactivation mechanism, suggesting that the alternative speculation described above is unlikely. Although our results indicate a structural similarity between p51/p63 and p53, the *p51/p63* mutations and the *p53* mutations displayed some differences in their ability to inactivate transactivation [*e.g.,* p51R204L *versus* p53R175L (15) and p51R313W *versus* p53R282W). These discrepancies might be attributable to slight differences in protein structure when expressed in yeast.

In this study, we investigated the transcriptional activities of *p51/p63* mutations in promoters containing p53-responsive sequences in yeast. The results of our biological assay suggested that both structural similarities and differences exist between p51/p63 and p53. The data shown in this study and in our previous report (19) suggest that the cellular signals of p51/p63 cross-talk partially, but not completely, with that of the p53 pathway (19). Recent studies on knock-out mice have shown that p51/p63 is required for limb and epidermal morphogenesis, and the phenotypes of these mice are different from those of p53 knock-out mice

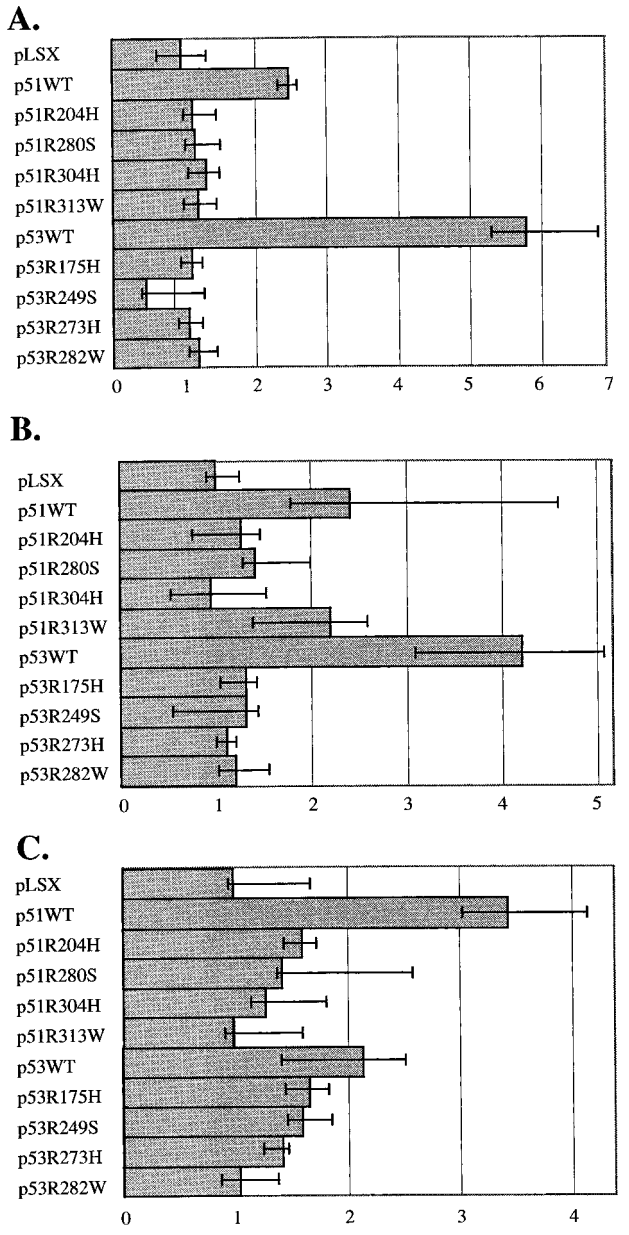

Fig. 3. Comparison of transcriptional activities for mutant p53 and mutant p51A/p63γ proteins in p53 target genes. *A, WAF1*; *B, MDM2*; *C, BAX* promoters. *X axis*, the green fluorescent intensity relative to a null expression vector (pLSX1). *Bars*, one SE of three values.

(25–27). These observations clearly indicate a difference in the biological functions of p51/p63 and p53. Both upstream and downstream p51/p63 signals must be further investigated to clarify the biological activity of p51/p63. Because yeast-based transcriptional assay is an artificial system, these data may not interpret instantly in mammalian cells. Further studies are also required to elucidate the pathogenic effects of tumor-derived p51/p63 mutations during tumorigenesis.

Note Added in Proof

Recently, new p51/p63 germline mutations were reported (J. Celli, *et al*., Cell, *99:* 143–153, 1999). Among the reported *p51/p63* missense mutations, some were detected at the residue of *p53* "hot spots," and functional assays of the missense mutation at the same residue were performed in this study.

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