Identification of a Novel Germ Line Variant Hotspot Mutant p53-R175L in Pediatric Adrenal Cortical Carcinoma

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Abstract
Hotspot mutations in the p53 tumor suppressor gene result in the disruption of DNA contact points or alter the overall structure of the protein to prevent DNA binding. When inherited, hotspot mutants are associated with Li-Fraumeni syndrome (LFS), a familial cancer predisposition. One of the most common hotspot mutations occurs at codon 175, resulting in an arginine to histidine substitution. We have identified a novel germ line variant of the 175 mutant (Arg to Leu; R175L) in a pediatric patient who developed adrenal cortical carcinoma. Surprisingly, the family is not tumor prone or associated with LFS. In vitro, the R175L mutant displayed an attenuated tumor suppressor activity in the regulation of transcription, colony formation, and apoptosis when compared with wild-type p53 and the R175H mutant. These findings suggest that p53-R175L retains sufficient activity to suppress LFS, but not adrenal cortical carcinoma. Therefore, not all hotspot mutants are functionally equivalent and the biochemical nature of the mutant may significantly influence clinical outcome. The implications of these results for genetic counseling are discussed. (Cancer Res 2006; 66(10): 5056-62)

Introduction
The p53 tumor suppressor plays a prominent role in the prevention of cancer by functioning as a transcription factor that induces downstream targets which negatively control cell growth (e.g., p21Waf1) and viability (e.g., Puma and Noxa; refs. 1–3). It is, therefore, not surprising that half of all human cancers have sustained inactivating somatic mutations in p53. Several residues are more frequently targeted than others and these are referred to as hotspot mutations (for review, see ref. 4). Interestingly, hotspot mutations disrupt critical DNA contact points or alter the structure in such a way that the protein no longer binds DNA in a sequence-specific manner (5). In either case, prototypic hotspot mutants are unable to efficiently induce target gene expression, cell cycle arrest, and cell death.

Germ line p53 hotspot mutations strongly predispose carriers to cancer as children or young adults. Indeed, epidemiology studies estimate that ~70% of males and 100% of females who inherit a p53 mutation will develop cancer, including tumors of the breast, brain, soft tissue, bone, blood, and adrenal cortex (6). The extraordinarily high occurrence of tumors associated with germ line p53 mutations is referred to as Li-Fraumeni syndrome (LFS); ref. 7). LFS is formally defined by a proband with a sarcoma before the age of 45 years and a first-degree relative with any cancer under 45 years of age, and an additional first-degree or second-degree family member with any cancer before 45 years or sarcoma at any age (8). Subsequent studies identified a variation of LFS, referred to as Li-Fraumeni-like (LFLS) syndrome, which includes a proband with any childhood cancer or a sarcoma, brain tumor, or adrenal cortical tumor before 45 years of age, and a first-degree or second-degree relative with a LFS-type tumor and an additional first-degree or second-degree relative with any cancer before 60 years of age (9). A telling feature of LFS and LFLS is a child who has developed an adrenal cortical tumor, although other mechanisms (e.g., Beckwith-Wiedemann syndrome) are also recognized to promote childhood adrenal cortical tumorigenesis.

Pediatric adrenal cortical tumors are extremely rare, with an annual worldwide incidence of 0.3 to 0.4 per million children under the age of 15 (10). Because childhood adrenal cortical tumors often arise within LFS and LFLS families, they are usually associated with a germ line p53 mutation (11). In many of these cases, the proband has inherited a hotspot p53 mutation. However, childhood adrenal cortical tumors can also occur outside the context of LFS/LFLS and be associated with a constitutional mutant p53 allele (12). For example, recent studies identified a group of pediatric adrenal cortical tumor patients from southern Brazil who inherited a mutation in exon 10, corresponding to an arginine to histidine substitution at amino acid 337 (R337H; ref. 13). Interestingly, the mutation occurs within the COOH-terminal oligomerization domain and not the DNA-binding region. Although the mutation could be tracked through multiple generations within the adrenal cortical tumor families, there were no reports of sarcoma or indications of an increased susceptibility to cancer in general. The R337H mutation strongly predisposes carriers to adrenal cortical tumors, but not to LFS or any other tumors (14). Interestingly, the histidine substitution at this site within the oligomerization domain alters the stability of the protein structure in a pH-dependent manner, which presumably forms the basis for the tumor specificity of this particular mutant (15).

The overall structure of p53 relies on arginine 175 (Arg175), which is located in the L2 loop of the DNA binding domain (5). Arg175 mediates the interaction between the L2 and L3 loops to maintain structural stability. Substitution of histidine for arginine at amino acid 175 (R175H) is one of the most common somatic mutations of p53 detected in human cancers (16, 17). The mutant R175H DNA binding domain is unable to bind specifically to the gadd45 promoter, is significantly less thermodynamically stable than the wild-type domain, and is predicted to be completely denatured at 37°C. Furthermore, full-length mutant p53 R175H...
lacks wild-type-like p53 function and is, therefore, transcriptionally inactive and unable to induce cell cycle arrest or apoptosis. Consequently, the R175H mutant is severely compromised in tumor suppressor function and when inherited is associated with LFS.

In this study, we identified a carrier of a novel germ line variant of the p53-R175H hotspot mutation, in which arginine is substituted for leucine, and developed pediatric adrenal cortical carcinoma (ACC). The R175L variant has been infrequently detected in sporadic cases (e.g., lung, liver, colon, and breast; 21 of ≥21,000 reported cases) and never before as a germ line mutation (17). Based on family history, the R175L germ line mutation is not associated with LFS or LFLS. We therefore investigated the in vitro and in vivo consequences of this mutation on p53 function and found that it retains partial activity in transcription and growth control. In contrast, the classical LFS hotspot mutant p53-R175H was completely defective in each assay. These findings show that not all amino acid substitutions, even when involving critical residues such as Arg175, have the same outcome with respect to function and tumor susceptibility. Notably, these findings bear important implications for genetic counseling and possibly clinical management.

Materials and Methods

DNA analysis. DNA was isolated from peripheral blood using the PureGene DNA Isolation system according to the manufacturer’s recommendations (Gentra Systems, Minneapolis, MN). Tumor DNA was extracted in xylene-100% ethanol (1:1) and digested in 50 mmol/L Tris-HCl (pH 8.0), and proteinase K (14 mg/mL) overnight at 37°C. Proteinase K was inactivated at 100°C for 5 minutes and the supernatant containing the DNA was collected after centrifugation. Whole blood DNA was quantified and amplified using the Affymetrix GeneChip p53 probe array, according to the manufacturer’s instructions. In addition, genomic DNA from whole blood and tumor samples were PCR-amplified and sequenced. Specifically, whole blood DNA was screened using primers spanning exons 5 and 6 of the p53 gene and genomic DNA from the paraffin-embedded tumor was screened using primers spanning exon 5 (primer sequences available on request). Whole blood DNA was purified by dialysis on 0.025 μm filter discs (Millipore, Bedford, MA) and diluted 1:50 in nuclease-free water. Tumor DNA was purified using ExoSAP-IT (U.S. Biochemical, Cleveland, OH) according to the manufacturer’s instructions. Both blood and tumor DNA were analyzed by high-throughput DNA sequencing (Hartwell Center) and compared with a human p53 genomic sequence (National Center for Biotechnology Information, accession no. U94788). p53 mutation status was also confirmed by DNA sequencing through the Carolinas Medical Center DNA Diagnostics Laboratory (Charlotte, NC).

Transactivation assays. Human SaOS-2 osteosarcoma cells and murine U94788 (10)1 fibroblasts (5 × 10^5 per 10 cm dish) were grown in DMEM containing 10% fetal bovine serum, penicillin (50 μg/mL), streptomycin (100 units/mL), and 2.5 mmol/L glutamine (complete DMEM) in 100 mm tissue culture dishes at 37°C under 5% CO2. The cells were cotransfected in duplicate with 250 ng p50-2Luc promoter-reporter and 1 μg cytomegalovirus (CMV)-Neo Bam (vector only), or CMV-Neo-Bam expressing wild-type p53 (WTp53), R175L, or R175L. The cells were lysed after 72 hours and protein yields determined using the bichinchoninic acid protein assay (Pierce, Rockford, IL). Normalized samples were analyzed by the single luciferase assay according to the manufacturer’s recommendations (Promega, Madison, WI).

Apoptosis assay. Human H1299 lung adenocarcinoma cells and human SaOS-2 osteosarcoma cells were grown at 4 × 10^5 cells/dish (35 mm glass coverslip-embedded dishes) in complete DMEM at 37°C under 5% CO2. Cells were microinjected with 50 ng/μl pGreenLantern (Life Technologies, Inc., now Invitrogen Corporation, Carlsbad, CA) which expresses green fluorescent protein, and coincubated with either 100 ng/μl CMV-Neo-Bam, WTp53, R175L, or R175H. Control injections included 100 ng/μl pGreenLantern only. Cells were microinjected using an Eppendorf transjector system with a Zeiss Axiovert 135TV microscope as previously described (18). Viable fluorescent cells were counted at specific time points after microinjection and photographed (Microinjection Core Lab, St. Jude Children’s Research Hospital).

Colony reduction assay. SaOS-2 cells were plated in duplicate in 100 mm tissue culture dishes and transiently transfected with 1 μg CMV-Neo-Bam, WTp53, R175L, or R175L. Cells were selected in G418 antibiotic (Invitrogen) at 0.8 to 1 mg/mL for up to 21 days. Colonies were washed briefly with PBS and fixed with methanol. Cells were stained with 1:20 Giemsa dye (Sigma-Aldrich, St. Louis, MO) for 45 minutes, washed briefly with distilled water and air-dried. Colonies were counted and photographed (Biomedical Communications, St. Jude Children’s Research Hospital).

Protein analysis. SDS-PAGE analysis was done with 30 μg protein/sample and the Novex NuPAGE Bis-Tris gel system (Invitrogen). Proteins were electrophoretically transferred to nitrocellulose membranes and blocked in TBS-T buffer [10 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 0.1% Tween 20] containing 5% nonfat milk. Membranes were hybridized with sheep polyclonal anti-human p53 Ab-7 (Calbiochem, San Diego, CA) diluted 1:2,500 in TBS-T with 5% nonfat milk at room temperature for 4 hours. Mouse monoclonal anti-human actin (Sigma) was diluted at 1:3,000. Membranes were washed with TBS-T and hybridized with either horseradish peroxidase–linked rabbit anti-sheep at 1:5,000 (Pierce) or horseradish peroxidase–linked sheep anti-mouse (Amersham Biosciences, Piscataway, NJ) at 1:3,000 for 1 hour at room temperature. The membranes were washed with TBS-T and developed with Supersignal West Dura Extended Substrate according to the manufacturer’s recommendations (Pierce).

Tissue preparation and immunohistochemistry. Adrenal samples were fixed in 10% neutral phosphate-buffered formalin and processed through graded ethanol to xylene and embedded in paraffin. Multiple 3 to 4 μm sections were cut and attached to lysine-treated glass slides. Heat-induced epitope retrieval was done with EDTA at pH 8.0. Tissue sections were placed in plastic Coplin jars containing preheated target retrieval solution (DAKO, Carpinteria, CA), heated in a household vegetable steamer (Sunbeam-Oster) for 35 minutes, and allowed to cool at room temperature for at least 15 minutes. Subsequent steps of the immunohistochemical staining procedure were done using the DAKO Autostainer at room temperature and included the following: (a) blocking of endogenous peroxidase in 3% H2O2 in PBS at pH 7.4 for 5 minutes; (b) blocking of nonspecific protein-binding sites using protein blocking solution (DAKO) for 5 minutes; (c) incubation with the primary p53 antibody (DAKO clone D0-7) for 1 hour; and (d) detection using the streptavidin-biotin-peroxidase based LSAB- kit (DAKO) for 2 × 15 minutes. 3,3′-Diaminobenzidine/H2O2 (Biogenex, San Ramon, CA) was used as the chromogen and hematoxylin as the counterstain. Samples that were positive for p53 are identified by the nuclear deposition of a permanent brown precipitate that is readily detected by light microscopy.

Results

Identification of the Germ Line p53-R175L Mutation Associated with ACC

An international pediatric adrenal cortical tumor registry and bank (IPACTRB) has been established at the St. Jude Children’s Research Hospital in order to centralize clinical information regarding the subjects and to collect tissue specimens. The registry component has been in existence since 1990 and has enrolled >250 subjects. The intention of the registry and bank is to broaden our understanding of the biology of adrenal cortical tumors and how to best treat the patients. The registry and bank have been approved by the St. Jude Children’s Research Hospital Institutional Review Board for the protection of human research subjects.

Recently, a female patient from North America who developed ACC at 3 years of age was enrolled in the IPACTRB. Pediatric ACC is often associated with LFS; however, the proband’s family history does not fulfill LFS or LFLS criteria (Fig. 1). Indeed, there was only one other case of cancer within this family, which was a uterine
tumor at 38 years of age (paternal grandmother). Uterine cancer is not an LFS tumor type and is rarely associated with somatic p53 mutations (61 cases of 21,512 total tumors; ref. 17). Nevertheless, given the strong association between p53 mutations and pediatric ACC, the germ line status of p53 in the proband was initially determined by Affymetrix p53 chip array analysis using genomic DNA prepared from peripheral blood leukocytes. Affymetrix analysis revealed a single point mutation (CGC to CTC) in exon 5 at codon 175, resulting in an arginine to leucine substitution (R175L; data not shown). DNA sequence analysis confirmed the Affymetrix results and showed the presence of the germ line wild-type and mutant p53-R175L alleles (Fig. 2). The father and two siblings of the proband are also carriers of the R175L mutation (data not shown), but none of these individuals developed cancer. These findings indicate that the R175L mutation may predispose carriers to adrenal cortical tumors without causing LFS.

**Elevated Expression of Mutant p53-R175L in ACC**

A hallmark of tumor suppressor genes that have been targeted in human cancers is the elimination of the wild-type allele with retention of the mutant allele, which is referred to as loss of heterozygosity. Particularly for the p53 tumor suppressor, the mutant allele is usually expressed and the missense protein accumulates to high levels in the nucleus. However, DNA sequence analysis showed that the adrenal tumor of the proband did not undergo loss of heterozygosity and retained both the wild-type and mutant allele (Fig. 2B). No second site mutations were detected throughout the coding region. This result is rare but not unfounded in pediatric adrenal cortical tumors harboring low-penetrance p53 mutations, as described by Varley et al. (12). As expected for a tumor carrying a p53 mutation, the p53 protein was well expressed and accumulated within the nucleus as shown by immunohistochemical analysis (Fig. 2C). In contrast, an
adrenal cortical tumor with wild-type p53 alleles failed to stain for p53 protein expression, consistent with the predicted short half-life of the protein. Taken together, these results support the concept that mutant p53-R175L is functionally impaired within the adrenal cortical tumor.

**In vitro Characterization of Mutant p53-R175L Function**

**Transactivation.** The consequence of the R175L mutation on p53 function was explored under a variety of controlled conditions. First, the ability of the R175L mutant to transactivate a p53-responsive promoter was tested using the p50-2Luc reporter plasmid, which contains p53 DNA-binding elements from the murine muscle creatine kinase promoter (19). Human osteosarcoma SaOS-2 cells and murine (10)1 fibroblasts, both p53-null cell lines, were cotransfected with p50-2Luc and either CMV-Neo-Bam empty vector (CMV only) or CMV-Neo-Bam-based vectors expressing human wild-type and mutant p53 proteins. The R175H mutant serves as a negative control because it is well established that it exhibits a denatured conformation and is severely defective in DNA binding and transactivation functions (20). As expected, wild-type p53, but not mutant p53-R175H, strongly induced promoter-reporter activity in SaOS-2 cells (Fig. 3) and (10)1 fibroblasts (data not shown). Mutant p53-R175L retained partial function and up-regulated the promoter at ~20% to 40% efficiency of that observed for wild-type p53 (Fig. 3). Western blot analysis showed relatively equal expression of each form of p53, indicating that the differences in activity reflect alterations in function and are not due to variations in protein levels (Fig. 3). Similar results were obtained when using 100 ng of each vector (data not shown). These findings show that mutant p53-R175L exhibits low, but significant transactivation function.

**Growth suppression.** To test the effect of the R175L mutation on growth suppression, SaOS-2 cells were transfected with CMV-only or CMV vectors expressing either wild-type or mutant p53. Cells were selected for antibiotic resistance in 1 µg/mL of G418 for 2 to 3 weeks, stained with Giemsa dye and photographed. As expected, cells transfected with CMV-only readily formed colonies, whereas wild-type p53 efficiently suppressed colony formation (Fig. 4). Mutant p53-R175H enhanced the number and size of the colonies at 1 µg DNA, consistent with its reported gain-of-function activity that promotes cell growth and survival (20, 21). By contrast, mutant p53-R175L markedly reduced colony formation, approximately equivalent to wild-type p53. Likewise, the R175L mutant was capable of suppressing colony cell growth when 100 ng of vector was employed (data not shown). Taken together, these results show that mutant p53-R175L maintains considerable growth suppressive activity.

**Apoptosis.** Induction of apoptosis by wild-type p53 is thought to be its primary tumor suppressor function. To determine whether mutant p53-R175L is competent for inducing programmed cell death, SaOS-2 and p53-null human lung adenocarcinoma H1299 cells were microinjected with CMV-only or CMV vectors expressing wild-type or mutant p53 proteins. A vector that expresses green fluorescent protein (pGreenLantern) was included to identify productively injected cells. Cells microinjected with the green fluorescent protein– and CMV-only plasmids maintained viability and increased in number over a 72-hour period (Fig. 5). In contrast, cells expressing wild-type p53 displayed hallmark features of apoptosis and detached from the plate, resulting in a significant loss of viability. Mutant p53-R175H and p53-R175L were essentially indistinguishable and had no effect on survival or cell number. Similar findings were obtained using human H1299 lung carcinoma cells (data not shown). Therefore, mutant p53-R175L exhibits a clear defect in triggering apoptosis.

**Modeling Structural Effects of the R175L Substitution**

The residues within the L2 (residues 163-195) and L3 (residues 236-251) loops of the DNA binding domain of p53 coordinate for two to three weeks, stained with Giemsa dye and photographed. As expected, cells transfected with CMV-only readily formed colonies, whereas wild-type p53 efficiently suppressed colony formation (Fig. 4). Mutant p53-R175H enhanced the number and size of the colonies at 1 µg DNA, consistent with its reported gain-of-function activity that promotes cell growth and survival (20, 21). By contrast, mutant p53-R175L markedly reduced colony formation, approximately equivalent to wild-type p53. Likewise, the R175L mutant was capable of suppressing colony cell growth when 100 ng of vector was employed (data not shown). Taken together, these results show that mutant p53-R175L maintains considerable growth suppressive activity.

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Zn$^{2+}$ which, in turn, maintains key residues within L3 in a conformation competent for binding DNA (5). Arg$^{175}$ lies within the L2 loop and plays a role in orienting Cys$^{176}$ and His$^{179}$, also within L2, for Zn$^{2+}$ binding. Furthermore, Arg$^{175}$ may form a salt bridge with Asp$^{184}$ to stabilize the L2 conformation and hydrogen bond with residues in L3 to stabilize the L2/L3 interface. It is well established that the R175H substitution significantly destabilizes the p53-native conformation, which exposes an epitope (monoclonal Ab240 reactivity) that usually only becomes accessible when the protein is denatured. This may arise because the His$^{175}$ side chain provides an alternative ligand for Zn$^{2+}$, disrupting the loop L2 conformation.

We have used computerized modeling to infer how the Leu$^{175}$ mutation may lead to altered stability of loop L2, and thus, influence the conformation of the entire DNA binding domain. A structural view of one wild-type p53 DNA-binding domain protomer from the crystal structure with DNA is presented in Fig. 6. The Zn$^{2+}$ ion which is tightly bound and required for DNA-binding activity is tetrahedrally coordinated by the side chains of Cys$^{176}$ and His$^{179}$ in loop L2, and Cys$^{238}$ and Cys$^{242}$ in loop L3 (22). In this p53 protomer, the side chain of Arg$^{175}$ projects from one side of the L2 loop toward that of Asp$^{184}$ on the other side and may participate in a stabilizing electrostatic interaction (Fig. 6B). Furthermore, the conformation of the Arg$^{175}$ guanidinium group may be further stabilized by hydrogen bonds with backbone atoms of Pro$^{191}$ in L2 and Met$^{237}$ in the L3 loop. In contrast, in the Leu$^{175}$ mutant, the shorter, uncharged side chain is unable to participate in the salt bridge or in hydrogen bonding to residues in L2 and L3, and may destabilize the native structure by creating a void at the L2/L3 interface (Fig. 6C). Although the loss of these interactions may destabilize the conformation in the vicinity of the L2/L3 interface, their loss would not necessarily disrupt the overall conformation of the DNA binding domain. The retention of significant transactivation and growth suppression activities indicates that this must indeed be the case.

**Discussion**

We have identified a family that harbors a novel germ line variant hotspot mutation, p53-R175L. Although the R175L mutation has been previously reported in the literature, albeit rarely (21 cases in >21,000 samples), this is the first incidence of it occurring as a germ line mutation (17).
The typical mutation at codon 175 results in an arginine to histidine substitution (R175H), which is the third most common somatic and germ line p53 mutation listed in the p53 database (17). A wealth of evidence shows that the R175H mutant is structurally unstable and functionally inactive. Indeed, it no longer binds DNA, transactivates target genes, or induces cell cycle arrest and apoptosis (20). Rather, it acts as a dominant-negative factor that inactivates wild-type p53 and cooperates with activated Ras to transform cells. Carriers of an inherited R175H mutation are associated with LFS and are at a remarkably high risk for developing a wide spectrum of tumors at a young age (22). Interestingly, one other inherited variant hotspot codon 175 mutation, R175G, has been previously reported (23). This mutation is also associated with LFS and predisposes carriers to early onset cancers. Functional studies in a yeast reporter system showed that the R175G mutant was equally defective in transactivation as the R175H hotspot mutant (23). By contrast, the R175L mutation identified here exists within a family that is not predisposed to cancer. In fact, the only tumor, in addition to the ACC, that occurred on the affected side of the family was a uterine cancer (paternal grandmother, 38 years of age; Fig. 1), which is not a tumor type involved in LFS or spontaneous tumors associated with p53 mutations (17). The p53 status of the paternal grandfather was wild-type, implying that the mutant allele was derived from the paternal grandmother. It should be noted that the father, who is a carrier, developed multiple lipomas. In addition, a second cousin from the affected side of the family also developed a benign soft tissue mass in his forearm at 11 years of age. Other low-penetrant germ line p53 alleles have been identified that increase the risk of adrenal cortical tumors without causing LFS or LFLS (12, 13). Analysis of this class of mutants generally shows that they are also impaired in activity, in contrast to the complete inactivation of function seen with classical hot spot mutants.

Our characterization of the adrenal cortical tumor provides additional evidence that mutant p53-R175L is functionally compromised in vivo and contributes to tumorigenesis. DNA sequence analysis of the tumor shows that p53 has not selected against the wild-type allele (Fig. 2). It has been shown that heterozygous p53 mutations occur in pediatric ACC without the incidence of a second site mutation (12). In addition, the missense protein is expressed at elevated levels in the nucleus of the adrenal cortical tumor cells (Fig. 2C). Intuitively, mutant p53 must not be sufficiently active for the tumor to tolerate nuclear accumulation of p53-R175L and to survive and proliferate. The apparent low tumor penetrance associated with the R175L mutation, especially in comparison with the R175H mutant, may reflect the partial activity of R175L in transactivation and growth suppression (Figs. 3 and 4). The R175L mutant has also been shown by others to transactivate the p21 promoter, but is unable to transactivate the Bax and IGF-BP3 promoters (24, 25). These findings, taken together with the results presented here, show that R175L controls partial tumor suppressor function. Loss of further activity would likely predispose carriers of the R175L mutation to LFS or LFLS syndrome.

Other somatic mutations at codon 175 have been observed in human tumors. One such example is R175P. Functional studies showed that R175P is defective in apoptosis, but less so in cell cycle arrest (26). The R175P mutant was critically assessed by generating a mouse knock-in mutation model. Thymocytes from homozygous mutant R175P mice are completely resistant to DNA damage–induced apoptosis, but cells can undergo cell cycle arrest to some extent. The mice are tumor prone, but tumor onset is delayed compared with p53 knockout mice, demonstrating that the R175P also retains a substantial degree of activity.

Mutant p53-R175H associates with hsp70 and binds to monoclonal antibody PAb240 (recognizes the denatured conformation) but not PAb1620 (specific for the native structure), indicating that the missense protein is unfolded (20, 24). By contrast, the p53-R175L mutant does not associate with hsp70 and is positive for binding to PAb1620 and not PAb240 (24). These findings suggest that the leucine substitution at codon 175 is less disruptive to the native p53 conformation, which is consistent with our data demonstrating that mutant p53-R175L displays significant transactivation function and contributes to growth suppression, as well as being associated with a less penetrant tumor phenotype in carriers.

Here, we have identified a naturally occurring human p53 germ line hotspot variant mutation, which is indeed a rare occurrence. Although we cannot reach a firm conclusion regarding the penetrance of the R175L mutation due to human subject consent issues, our data indicate that the R175L variant exhibits a milder phenotype than the LFS-associated germ line R175H and R175G mutations. It is becoming increasingly clear that the biochemical nature of the amino acid substitution at a particular residue, including hotspot mutants, could have very different clinical outcomes. Germ line R337H mutations selectively predispose carriers to childhood adrenal cortical tumors, whereas the R337C mutant is associated with breast cancer and LFLS syndrome (13, 27, 28). Similarly, the R175H hotspot mutation causes LFS, whereas our results to date indicate that the R175L can cause ACC without being associated with LFS or LFLS syndrome. Although a number of knock-in mutant p53 mouse models have been recently generated to test the effects of different amino acid substitutions in a selected site (26, 29, 30), the identification of the R175L mutation in a family with a child who developed an adrenal cortical tumor offers a unique opportunity to study this phenomenon in a human setting. It will be important to continually monitor these individuals in the future to fully evaluate the effect of the germ line R175L mutation on tumorigenesis. Finally, the information provided by the genetic and biochemical data presented in this article shows the importance of thorough screening for p53 status in cancer patients and their families for use in diagnosis, treatment, and genetic counseling.

Acknowledgments

Received 12/22/2005; revised 2/15/2006; accepted 3/3/2006.

Grant support: NIH/National Cancer Institute CA63230 and CA71907 (G.P. Zambetti), CA104568 (R.W. Kriwacki), Cancer Center CORE grant CA21765, and by the American Lebanese Syrian Associated Charities.

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We thank Jin Ling Wang, Simon Moshiach, and Dr. Jill Lahti for their technical assistance, as well as the Hartwell Center for the Affymetrix p53 gene chip and DNA sequence analyses.

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