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Towards an understanding of the role of p53 in Adrenocortical Carcinogenesis

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Abstract

Adrenocortical carcinoma (ACC) is recognized to be a component tumor of the Li Fraumeni Syndrome (LFS), a familial cancer predisposition resulting from germline mutations in the p53 tumor-suppressor. p53 activity is tightly regulated by multiple post-translational mechanisms, disruption of which may lead to tumorigenesis. ACC is present in disproportionately high rates among p53-mutation carriers, suggesting tissue-specific manifestations of p53 deficiency. Additionally, p53-associated ACC demonstrates a strong predominance in infants and children. Several of the p53 alleles associated with pediatric ACC, however, retain significant wild-type activity and demonstrate incomplete penetrance, a finding distinct from other LFS-component tumors. In this review, we discuss the relationship between p53 and adrenocortical carcinogenesis, with specific focus on disease-specific alleles, tumorigenesis in the context of adrenal development and potential therapeutic approaches to p53-associated ACC.

1. Introduction

Lesions in the p53-tumor suppressor signaling pathway play a fundamental role in the pathogenesis of adrenocortical carcinoma (ACC). Similarly, germline mutations in p53 are associated with development of the Li-Fraumeni Syndrome (LFS, OMIM #151623), an autosomal-dominant cancer predisposition syndrome. The overlap between the two diseases was first recognized in 1988 when Li(Li, Fraumeni, Mulvihill et al., 1988) and colleagues identified ACC to be a component tumor of the LFS. It would be a further two years before the association between LFS and p53 was established (Malkin, Li, Strong et al., 1990). Since then, much has been learned regarding the relevance of p53 to development of

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adrenocortical carcinoma, although many unanswered questions remain. This review will highlight critical aspects of the role of p53 in ACC epidemiology and biology and will place these data within a clinical context with implications for disease presentation, prognosis and treatment.

The *p53* gene encodes a multi-domain homo-tetrameric transcription factor with well over 100 direct transcriptional target genes identified to date (Riley, Sontag, Chen et al., 2008). It is the most frequently altered gene in sporadic cancers, with greater than 50% of human tumors harboring somatic mutations (Hainaut and Hollstein, 2000, Hollstein, Moeckel, Hergenbahn et al., 1998). The p53 protein is comprised of multiple functional domains (Figure 1). The N-terminus (amino acid residues (aa) 1-42 and 43-62) serves as a transcriptional activator, while a central 200 amino acid domain binds DNA at cognate recognition sequences within regulatory regions of target genes (Hainaut and Hollstein, 2000). Finally, the C-terminus (aa 325-356) is required for tetramerization and contains multiple nuclear localization sequences as well as multiple lysine residues which are key to regulating p53 activity.

p53 has central roles in mediating the cellular response to genotoxic stress (such as ionizing radiation, free radicals, hypoxia and metabolic challenge) and oncogene activation, and fulfills these roles by activating pathways involved in cell-cycle arrest and DNA-damage repair. These responses to cellular stress are postulated to afford the cell the opportunity to respond to the stress and to repair accrued DNA damage. Failing this, and in circumstances of catastrophic genomic compromise, however, p53 induces apoptotic pathways, principally via PUMA (p53-upregulated modulator of apoptosis) to abrogate propagation of a corrupted genome. In addition to these roles, p53 is known to mediate angiogenesis, cellular senescence and has recently been recognized to participate in the inflammatory response and in suppressing invasiveness (Elyada, Pribluda, Goldstein et al., 2011, Frank, Leu, Zhou et al., 2011, Goodman, Hofseth, Hussain et al., 2004, Portwine, 2000, Schetter, Heegaard and Harris, 2010, Zheng, Lamhamedi-Cherradi, Wang et al., 2005).

2.1 Regulation of p53 - a fine balance

To fulfill its role as “guardian of the genome” (Lane, 1992), p53 coordinates a wide array of cellular processes and, as a result, is reliant on a meticulously refined network of positive and negative regulators. Levels of p53 under steady-state, unstressed conditions are maintained at low levels, kept in this state by constitutive repression. In the face of cellular stress, this repression is lifted and p53 is stabilized and activated.

While regulation of p53 levels and activity are intricate processes and are extensively reviewed elsewhere (Boehme and Blattner, 2009, Dai and Gu, 2010, Hock and Vousden, 2010, Kruse and Gu, 2009, Vilborg, Wilhelm and Wiman, 2010), this review will consider only a few key components of this process. Arguably the most important regulator of p53 is the E3-ubiquitin ligase, MDM2 (or the human homolog, “HDM2”) which acts to regulate p53 levels, cellular localization and activity. MDM2 binds p53 and targets it for either cytosolic translocation (via monoubiquitination) or proteasomal degradation, when polyubiquitinated (Lee and Gu, 2010). MDM2 is a transcriptional target of p53, and thus represents a key node in a negative feedback loop. Elevated levels of MDM2 result in loss of p53 activity and propensity for tumor formation. MDMX (also known as MDM4) is similarly able to bind p53, but lacks intrinsic ubiquitin ligase activity. In contrast to MDM2, MDMX expression is not regulated by p53. MDMX is able to bind MDM2 and the heterodimer can interact with p53 to effect its degradation. Similarly, MDM2/MDMX heterodimers bound to p53 interfere with transcriptional activation (Ohkubo, Tanaka, Taya et al., 2006). Both MDM2- and MDMX- deficient mice are embryonic lethal, presumably due to massive overexpression of p53. The embryonic lethality of each null, as well as an

MDM2/MDMX double knockout are rescued by a *p53* null background (Jones, Roe, Donehower et al., 1995, Montes de Oca Luna, Wagner and Lozano, 1995). Similarly, the embryonic lethality of a transgenic *MDM2* mouse deficient in E3 ubiquitin ligase activity is rescued by loss of *p53* (Itahana, Mao, Jin et al., 2007). These studies highlight the meticulous dose balance between these signaling molecules and the integral role of MDM2 and MDMX in repression of p53 at physiologic steady-state.

In addition to control of activity by MDM2 and MDMX, direct post-translational modification of the p53 protein at multiple sites, including acetylation, methylation, phosphorylation and neddylation modulate the activity of p53 by fine-tuning its stability, its ability to bind DNA, and recruitment of transcriptional cofactors at promoters of target genes (Dai and Gu, 2010, Hollstein and Hainaut, 2010). Dispersed throughout the protein, although relatively enriched at the N- and C-termini are multiple serine/threonine residues targeted by a variety of intracellular kinases. For example, in response to DNA damage by chemotherapeutic agents, irradiation or reactive oxygen species, activation of ATM/ATR and CHK2 results in phosphorylation of key serine residues within the N-terminus of p53 including Ser 15 and Ser20. Increasing phosphorylation at serine and threonine residues within the N-terminus tips the balance towards binding of the transcriptional co-activator CBP/p300, which displaces the repressor MDM2, thereby directing the cell to a state of de-repression and transcriptional activation (Teufel, Bycroft and Fersht, 2009).

To date, nine lysine residues within p53 have been identified as acetylation sites and the relative balance of histone acetylases (HATs) and histone deacetylases (HDACs) controls the acetylation balance of the protein. Progressive acetylation biases p53 towards a more active, stable state by interfering with MDM2/MDMX complex formation, blocking ubiquitination and allowing for activation of transcriptional cofactors.

2.2 Polymorphic Variants May Modulate p53 Signaling

Further alterations in p53 activity may be influenced by polymorphisms both within the *p53* gene itself and in its regulatory proteins, such as MDM2. Although numerous SNPs and sequence variants have been identified within *p53* (see Figure 3 from ref (Whibley, Pharoah and Hollstein, 2009)), few have been associated with alterations in disease risk or course. To date, 19 exonic polymorphisms have been identified, representing <10% of polymorphisms at the *p53* locus.

Of the few polymorphisms that have been associated with disease, the most studied has been a single-nucleotide polymorphism at codon 72, which results in the substitution of an arginine for a proline residue. Although the proline allele appears to be the ancestral form, based on conservation in non-human primates, the arginine allele exceeds a frequency of 50% in some genetic backgrounds, including the Yoruban population in Nigeria (Altshuler, Gibbs, Peltonen et al., 2010). While multiple *in vitro* transfection-based assays have demonstrated differences between the two alleles in their ability to induce apoptosis, cell-cycle arrest or DNA repair, alterations in human tumor cells are less conclusive (den Reijer, Maier, Westendorp et al., 2008, Dumont, Leu, Della Pietra et al., 2003, Salvioli, Bonafe, Barbi et al., 2005, Thomas, Kalita, Labrecque et al., 1999). Several association studies have implicated the codon 72 polymorphism in susceptibility to cervical, lung, breast and other cancers. Nonetheless, Whibley and colleagues aptly point out that none of the many genome-wide association studies of breast, gastric, lung, colorectal, prostate or other cancers have demonstrated statistically-significant associations with the codon 72 SNP. While there are hints of association with disease susceptibility, prognosis or response to therapy, none of the extant studies offer definitively robust, clinically significant data. It remains, possible, however, that the codon 72 residue may serve as a disease-modifying polymorphism in cooperation with other variants either in *p53* or other loci.

Similarly a 16-bp insertion within intron 3 (PIN3) (in close proximity to the codon 72 polymorphism) has been associated with an increased risk of several cancers (Boldrini, Gisfredi, Ursino et al., 2008, Costa, Pinto, Pereira et al., 2008, Gemignani, Moreno, Landi et al., 2004, Wang-Gohrke, Weikel, Risch et al., 1999), as well as a predilection for earlier onset tumors. This allele may be associated with alterations in mRNA processing, resulting in decreased *p53* transcript levels, accounting for its biological effect (Gemignani et al., 2004).

The Pro47Ser polymorphism has been reported only in patients of African origin and is present in that population at an allele frequency of 5-7% (dbSNP) (Almeida, Custodio, Pinto et al., 2009, Altshuler et al., 2010, Li, Dumont, Della Pietra et al., 2005, Pinto, Yoshioka, Silva et al., 2008). This variant is adjacent to phosphorylation targets of p38 and HIPK2 (Bulavin, Saito, Hollander et al., 1999, Cecchinelli, Porrello, Lazzari et al., 2006, Rinaldo, Prodosmo, Mancini et al., 2007, Takekawa, Adachi, Nakahata et al., 2000) and may thus influence phosphorylation at this site, consequently altering the *p53*-mediated transcription of pro-apoptotic genes, as has been reported (Li et al., 2005).

Finally, a single-nucleotide polymorphism within the first intron of MDM2 (SNP309), has been demonstrated to influence expression of MDM2 (and, based on the negative feedback loop, have an opposite effect on *p53* levels) (Bond, Hu, Bond et al., 2004). Among patients with LFS, carriers of the SNP309G variant develop tumors at an accelerated rate (Bond, Hu and Levine, 2005, Bougeard, Baert-Desurmont, Tournier et al., 2006, Ruijs, Schmidt, Nevanlinna et al., 2007, Tabori, Nanda, Druker et al., 2007).

Other variants in *p53* and *p53*-pathway genes and response elements are reviewed elsewhere (Whibley et al., 2009).

Clearly the multiple levels of regulation of *p53* orchestrate a fine balance of activity via myriad convergent mechanisms, many of which are proving to be attractive therapeutic targets in *p53*-associated tumors (see below).

3.1 Epidemiology of *p53*-associated ACC

Since the identification of ACC as a component tumor of the LFS, much work has gone into characterizing the landscape of *p53* mutations within the germline and tumors of affected individuals. An indispensable resource in this regard has been the work of Hainaut and colleagues in compiling an extensive worldwide database of both germline and somatic *p53* mutations based on reports from the published literature and generalist databases, currently in its 15th revision (www-p53.iarc.fr) (Petitjean, Mathe, Kato et al., 2007). Examination of the data contained within this database reveals an excess of adrenocortical tumors in carriers of germline *p53*-mutations in comparison to their rate in the general population. Among the former group, ACCs comprise 11.9% of all tumors (n=1165), constituting the 4th largest tumor type in this population (behind breast, soft-tissue and brain tumors). In contrast, in the general population, ACCs represent <1% of diagnosed cancers and, when considered amongst all non-thyroidal endocrine tumors, are the 40th most common cancer (American Cancer Society, 2010). A tissue-specific effect of *p53*-loss is thus likely relevant to the pathogenesis of ACC.

Several studies have demonstrated that the age-of-diagnosis of adrenocortical carcinomas in the general population follows a bimodal distribution with peaks in the first and fifth decades of life (Wajchenberg, Albergaria Pereira, Medonca et al., 2000, Wooten and King, 1993). Examination of the most recent Surveillance, Epidemiology and End-Results (SEER, 2011) data confirm these reports, albeit with a second incidence peak extending into the 7th decade (Figure 2A). In contrast, ACC among individuals with *p53* germline mutations

follows a unimodal distribution with 68% of individuals presenting in the first 4 years of life and 92% of patients presenting in the pediatric age-group (Figure 2B). Although these latter data may be somewhat biased by the historical lack of *p53* testing in older subjects, they provide compelling evidence that ACC associated with *p53* germline mutations is predominantly a disease of infancy and early-childhood, a fact that may suggest inherent differences in the underlying biologic causes of the two peaks and may ultimately help explain the biologic origins of this disease.

3.2 Prevalence of *p53* germline mutation among individuals with ACC

Several series have attempted to establish the rate of germline *p53* mutations among individuals with ACC. In the first published study, 3 of 6 (50%) children with ACC (none of whom had a family history consistent with LFS) had a germline *p53* mutation (Wagner, Portwine, Rabin et al., 1994). Subsequently, in a study of 14 individuals in a local catchment area of a single institution, >80% carried a germline mutation in *p53*, with a strong representation of two recurrent alleles suggesting the possibility of a common ancestor (Varley, McGown, Thorncroft et al., 1999). More recently, Gonzalez et al. demonstrated that among 21 individuals with ACC referred to their facility for *p53* testing, 14 (67%) carried mutations in the *p53* gene. Only recently has the diagnosis of ACC alone, in the absence of a family cancer history, constituted sufficient indication for germline *p53* testing (Tinat, Bougeard, Baert-Desurmont et al., 2009) thus these data are inherently biased by enrichment for penetrant alleles, or those associated with the LFS and may overestimate the rate of *p53* aberrations in the total population of individuals with ACC. A true estimate of the prevalence of *p53* mutations would be obtained only through a sequential analysis of individuals with ACC unselected for family history.

The prevalence of *p53*-germline variation among the adult population with ACC is less clear. We are unaware of any published series that has documented the prevalence of germline *p53* mutations in adults with ACC, although Hermann and colleagues recently showed that 3 of 140 adults with ACC (2.1%) carried germline *p53* mutations (Herrmann L, Heinze B, Fassnacht M et al., 2011).

Relatives of children with ACC often have an elevated incidence of other cancers; however, absence of family history should not preclude assessment of *p53*-germline status in affected individuals. In fact, the association between ACC and *p53* germline mutations is compelling enough to warrant genetic counseling and consideration of *p53* analysis in all newly-diagnosed individuals, particularly those in the pediatric age group (Tinat et al., 2009, Varley et al., 1999).

3.3 Somatic mutations in *p53*

Estimates of the rate of somatic mutation of *P53* range from 20-70% of sporadic ACCs (Barzon, Chilosi, Fallo et al., 2001, Libe, Groussin, Tissier et al., 2007, Ohgaki, Kleihues and Heitz, 1993, Reincke, Karl, Travis et al., 1994). In an analysis of 36 adrenocortical carcinomas with LOH at 17p13, only 12/36 tumors carried *p53* mutations, although these tumors were significantly larger and were associated with a more advanced stage and shorter disease-free survival (Libe et al., 2007).

4. Adrenocortical Carcin-omics: Distinctions between pediatric and adult disease

Using comparative genomic hybridization, both Figueiredo and colleagues and James and colleagues separately identified a number of recurrent copy number changes among ACCs in children. These changes were independent of pathological diagnosis (adenoma vs.

carcinoma) and, in the latter study, of germline *p53* status of the individual (Figueiredo, Stratakis, Sandrini et al., 1999, James, Kelsey, Birch et al., 1999). The changes observed were distinct from genomic alterations identified among tumors from adults with ACC (Kjellman, Kallioniemi, Karhu et al., 1996, Zhao, Speel, Muletta-Feurer et al., 1999), a finding the authors speculated implied an embryonal origin of pediatric ACC. Additionally, Zhao and colleagues demonstrated that increasing numbers of chromosomal changes corresponded to increasingly malignant behavior of the tumors (Zhao et al., 1999).

More recent studies using array CGH analysis demonstrated that the rate of copy number variation (CNV) within the healthy population was remarkably invariant. Among individuals with germline *p53* mutations, however, a substantial increase in CNV was noted and this was further increased amongst *p53* heterozygous individuals with a history of cancer (Shlien, Tabori, Marshall et al., 2008). Furthermore, specific CNVs have been correlated to survival rates among adults with ACC (Stephan, Chung, Grant et al., 2008). Knowing that $\geq 50\%$ of children with ACC harbor germline mutations in *p53*, it would thus stand to reason that these children have increased rates of copy number variation and that specific variants may explain development of disease in individuals with low-penetrance *p53* alleles (Varley et al., 1999, Zambetti, 2007) and may also explain the heterogeneity of disease among individuals with a similar genetic background (Figueiredo, Sandrini, Zambetti et al., 2006).

Multiple studies have examined the ACC transcriptome and have demonstrated expression profiles that clearly distinguish ACC from adrenocortical adenomas (Giordano, Kuick, Else et al., 2009, Giordano, Thomas, Kuick et al., 2003, Laurell, Velazquez-Fernandez, Lindsten et al., 2009, Slater, Diehl, Langer et al., 2006). Furthermore, comparison of pediatric and adult ACCs revealed unique profiles, with the pediatric tumor transcriptome bearing significant similarity to that of fetal adrenal tissue (West, Neale, Pounds et al., 2007). De Reynies et al. used transcriptome analysis to demonstrate upregulation of the *p53* pathway in ACC (de Reynies, Assie, Rickman et al., 2009). Further analysis was able to identify a unique transcriptomic signature of tumors bearing an inactivating mutation of *p53* (Ragazzon, Libe, Gaudoux et al., 2010).

Transcriptional profiling has demonstrated distinct signatures of adult and pediatric ACCs (West et al., 2007). Taken together with the biologic and molecular distinctions between childhood and adult ACC as described by Almeida elsewhere in this issue, and the epidemiological differences, these findings suggest that pediatric and adult ACC may represent genetically and biologically distinct entities with convergent pathology.

5.1 *p53* alleles in ACC

The vast majority of mutations in *p53* are missense mutations (Hainaut and Hollstein, 2000). These mutations are dispersed at non-random sites. In fact, a handful of common “hotspot” mutations (at codons 175, 245, 248, 249 and 273, 282) account for $\sim 20\%$ of all reported mutations in *p53*. Roughly 80% of all mutations are located within the core DNA-binding domain of the protein and lead to inactivation via conformational effects (eg. the common R175H mutation) or by disrupting the ability of the protein to directly bind DNA (eg. the R273H mutation). A review of germline mutations amongst individuals with ACC from the IARC database demonstrates a similar clustering of mutations within the DNA-binding domain (Figure 3).

p53 binds DNA as a homo-tetramer. In a heterozygous cell, presence of a mutant protein unable to bind DNA thus exerts a dominant-negative effect by oligomerizing with WT proteins and excluding the complex from DNA binding and transcriptional activation. Indeed $>80\%$ of *p53* mutations are predicted to have a dominant-negative effect (Petitjean, Achatz, Borresen-Dale et al., 2007, Petitjean et al., 2007). In this circumstance the tumor cell

may be freed of the pressure to undergo loss of heterozygosity for the wild-type allele. Among mutations lacking the dominant-interfering activity, however, determination of LOH within the tumors remains an important component of the assessment of *p53* status (Dearth, Qian, Wang et al., 2007).

5.2 R337H: A unique and prevalent mutation in Southern Brazil

For several years, a 10-fold increased incidence of adrenocortical carcinoma had been observed in South-Eastern Brazil, in the states of Paraná, Santa Catarina and São Paulo. In almost all of these individuals a common germline mutation within the oligomerization domain of *p53*, p.R337H, was identified (Latronico, Pinto, Domenice et al., 2001, Ribeiro, Sandrini, Figueiredo et al., 2001). In contrast to mutations conventionally associated with the LFS, *in vitro* assays of the R337H allele demonstrate near wild-type function under physiologically normal conditions (Lomax, Barnes, Hupp et al., 1998, Ribeiro et al., 2001). The arginine residue at position 337 normally forms a salt-bridge with an aspartic acid at residue 352 of an adjacent monomer, stabilizing the *p53*-oligomer. Substitution of histidine at position 337 disrupts this interaction in a pH-dependent manner (DiGiammarino, Lee, Cadwell et al., 2002). At pH slightly greater than physiologic values, tetramer formation is disrupted and transcriptional activity of *p53* is diminished. The precise physiologic context in which this functional disruption affects tumor formation is still unclear, although elevations in pH in apoptotic cells may be a contributing factor. Similarly the unique biochemical environment of the adrenal cortex (which is dedicated to steroidogenesis), may further contribute to a tissue-specific effect, and may explain the reported predominance of adrenocortical tumors among individuals with this mutation (in contrast to other LFS-associated mutations) (Ribeiro et al., 2001).

Initially, the R337H mutation was thought to predispose carriers primarily to ACC; however, recently it has been identified in association with families meeting criteria for LFL (Li Fraumeni-like syndrome) (Achatz, Olivier, Le Calvez et al., 2007) as well as with individuals with breast cancer (Assumpcao, Seidinger, Mastellaro et al., 2008, Palmero, Schuler-Faccini, Caleffi et al., 2008), choroid plexus carcinoma and osteosarcoma (Custodio, Taques, Figueiredo et al., 2011, Seidinger, Mastellaro, Paschoal Fortes et al., 2011). Nevertheless, ACC constitutes the plurality of tumors identified in carriers of the R337H allele (Petitjean et al., 2007).

The penetrance of the allele has been estimated at approximately 10%. Remarkably, inheritance of this allele confers an estimated 20,000-fold increased risk for ACC (Figueiredo et al., 2006, Michalkiewicz, Sandrini, Figueiredo et al., 2004).

6. Escape from the LFS Shadow: What can we learn from Low-Penetrance Alleles?

The vast majority of ACC-associated *p53* mutations identified to date result in substantial loss of *p53* function. This may, in part, reflect an ascertainment bias inherent to the identification of index patients who were mostly selected for either family history of cancer and/or highly penetrant phenotypes. In studies not reliant on a family history of cancer, a number of low-penetrance alleles have been identified including the R337H allele (Figueiredo et al., 2006, Pinto, Ribeiro, Kletter et al., 2011, Ribeiro et al., 2001, Varley et al., 1999, West, Ribeiro, Jenkins et al., 2006). *In vitro* studies of several of these alleles, including R175L and R337H, have revealed a *p53* protein with retention of a substantial proportion of its wild-type activity.

Olivier and colleagues observed that *p53* germline mutations in individuals with ACC clustered at residues 151, 152, 219 and 220, residues located on the opposite surface from

the DNA-contacting surface of the protein (Olivier, Goldgar, Sodha et al., 2003). It has been speculated that these alleles may have a milder effect on p53 functions (Palmero, Achatz, Ashton-Prolla et al., 2010).

In a molecular tour-de-force, Kato and colleagues systematically generated a library of all possible missense mutations at each residue of the p53 protein. They subsequently used a yeast-based assay to assess the ability of each mutant to transactivate eight different p53-response elements from known target genes (Kato, Han, Liu et al., 2003). While these data are not always correlated with behavior in human cells (Kakudo, Shibata, Otsuka et al., 2005), they continue to provide a powerful tool to predict the consequences of a given mutation on p53 function. Consistent with the analyses of the R175L and R337H alleles, many of the other ACC-associated alleles are predicted, in this assay, to retain partial p53 function. This may suggest that while neoplastic transformation of many LFS-component tumor precursors require near-total loss of p53 function, more subtle decreases in p53 function may be sufficient to tip adrenocortical cells towards this fate. In other words, the adrenocortical carcinoma cell-of-origin may be more vulnerable to loss-of-function in p53 than other tumor progenitors.

Despite its initial identification in association with the LFS, only 39% of individuals with ACC in the IARC database are classified as meeting criteria for Li Fraumeni Syndrome or Li Fraumeni-Like syndrome (Pinto et al., 2011). In contrast, 79% of individuals with breast cancer, 75% of individuals with brain tumors and 78% of individuals with bone tumors are so classified, suggesting again that a subset of p53 mutations in ACC may have tissue-specific effects and may not predispose to other LFS component tumors.

In light of these observations, it becomes more challenging to the clinician to counsel patients regarding prognosis. Do the previous studies relating survival, recurrence risk, and risk of second malignancies among LFS patients apply to patients with less penetrant alleles? Is there a different malignant potential among tumors bearing one of the lower penetrance alleles? Does their presence suggest other modifier gene variants to promote tumorigenesis in a milder background? Are different treatment regimens or surveillance protocols indicated for individuals with milder alleles? Finally, what can we learn regarding the biology of the adrenal gland that sensitizes it to tumor formation in the setting of alleles that do not lead to tumorigenesis in other tissues?

7. Implications for adrenal biology-a vulnerable gland?

What ramifications do these findings have on the biologic origins of ACC? In particular, is there significance to the young age of presentation among p53-mutation carriers and/or the presence of low-penetrance alleles that preserve significant amounts of residual p53 activity?

Clues may reside in an understanding of the ontogeny of the adrenal gland. During fetal life, the adrenal cortex is composed of two distinct zones: the outer definitive zone will eventually form the multilayered adult cortex, while the inner fetal zone comprises approximately 85% of the volume of the fetal gland. The principal product of the fetal zone is dehydroepiandrosterone (DHEA) and its sulfate, are subsequently converted by placental aromatases to estrogens which help to maintain the pregnancy. At birth, the fetal zone undergoes a robust wave of apoptosis, leaving in its wake the definitive zone, which zonates into the adult cortex composed of the histologically and biochemically defined zona glomerulosa (responsible for mineralocorticoid production), zona fasciculata (glucocorticoid production) and zona reticularis (androgen synthesis).

The massive apoptosis that occurs during the regression of the fetal adrenal cortex over the first years of life, may thus transiently establish a vulnerable state, whereby perturbations in the apoptotic machinery (such as loss of p53) could interfere with the physiologic regression of fetal adrenal cells and predispose towards tumor formation although this remains to be demonstrated.

Is it a coincidence that >50% of pediatric adrenal tumors present with androgen excess, when a principal product of the fetal cells is DHEA, an androgen (Michalkiewicz et al., 2004)?

Given the strong association between ACC and *p53* mutations in children (and less so in adults), different transcriptional profiles, different clinical behavior and distinct molecular pathogenesis (see the review by Almeida in this issue), we propose that ACC in children represents a biologically distinct disease entity from that in adults, and that it results from failed regression of fetal cells, which may be predisposed by lesions in the apoptotic pathway, most commonly, mutations in *p53*. We further anticipate that analysis of those children with a *p53*^{wt} germline genotype, will identify germline lesions in other components of the *p53* and/or apoptotic pathways.

8. Is there a role for pre-symptomatic screening?

Recognition that an individual carries an inherited predisposition to cancer, and, in the case of the LFS, multiple primary cancers, obligates the clinician to consider what steps may be taken to mitigate morbidity, accepting that tumor development may be unavoidable. Estimates of tumor risk among *p53*-mutation carriers predict that 50% of individuals will develop cancer by age 30 and 90% by age 60 (Lustbader, Williams, Bondy et al., 1992). These estimates, however, are based on individuals defined by classic, stringent LFS criteria and may not be generalizable to more recent, broader definitions of the syndrome.

A recent report detailing a clinical surveillance protocol to monitor individuals with germline *p53* mutations demonstrated that among patients who underwent surveillance, tumors were detected at an earlier stage and tumor-associated morbidity and mortality were significantly decreased in comparison to a control population that did not undergo active organ-targeted screening (Villani, Novokment and Malkin, 2011). This finding provides compelling motivation for screening pre-symptomatic first-degree relatives of affected individuals, since identification of mutation-carriers may allow for early identification of tumors that would otherwise not come to diagnosis until an advanced stage.

These findings are certainly relevant to classic, highly-penetrant, *p53* alleles that result in substantial diminution of protein function. What, however, are the implications for individuals carrying *p53* alleles that appear to maintain significant residual function? At present, there is no evidence to suggest that these individuals are at increased risk for multiple primary malignancies, although numbers and duration of follow-up is currently limited. Similarly with penetrance as low as 10%, screening asymptomatic relatives for mutation stands to identify a large proportion of genetically-affected individuals who may never go on to develop disease. In the absence of a better understanding of disease modifiers and other elements of genetic background and environmental influences, it is challenging to offer reliable prognostic data to these individuals. Certainly, careful consideration should be given to the implications of a positive test and the decision to test asymptomatic individuals should involve extensive discussion between the patient(s), physician and genetic counselors.

9. Mouse models of human adrenocortical tumors

Genetically-engineered mice can serve as important models for studying human diseases, especially rare cancers such as ACC. Indeed, the physiological relevance of oncogenes, tumor suppressors and signaling pathways that are deregulated in human ACC can be challenged in transgenic and knockout mice. Several murine models of p53 dysfunction have been generated, including p53-mutant overexpression (Lavigne, Maltby, Mock et al., 1989), knock-out (Donehower, Harvey, Slagle et al., 1992, Jacks, Remington, Williams et al., 1994) and targeted substitutions resulting in mutant proteins replicating those identified in humans with LFS (Lang, Iwakuma, Suh et al., 2004, Olive, Tuveson, Ruhe et al., 2004). Like humans with germline *p53* mutations, these mice develop multiple tumors at young ages. Strikingly absent from the tumor profile of p53-deficient or mutant mice, however, are adrenocortical carcinomas, particularly given the excess of ACC in humans with *p53*-alterations. This absence may reflect the use of more severe alleles of *p53* in these models (either complete loss, or non-functional alleles with dominant-negative activity). Additionally, the absence of ACC in mouse models of p53 may reflect differing adrenal physiology between humans and mice. The latter, for example lack adrenal 17 α -hydroxylase expression and are therefore unable to synthesize adrenal androgens, thus establishing a different biochemical milieu (Keeney, Jenkins and Waterman, 1995, van Weerden, Bierings, van Steenbrugge et al., 1992).

There are, however, a number of murine models of adrenocortical carcinogenesis. For example, the transcription factor SF1 is commonly amplified and overexpressed in pediatric adrenocortical carcinoma. Consistent with these findings, several lines of transgenic mice expressing varying levels of SF1 develop adrenocortical hyperplasia and fully penetrant tumors of the gonadal phenotype in a dose-dependent manner (Doghman, Karpova, Rodrigues et al., 2007). Similarly, dysregulation of the Wnt pathway has been frequently observed in human adrenocortical adenomas and carcinomas, and overexpression of constitutively active β -catenin specifically in the mouse adrenal cortex results in hyperplasia, dysplasia and malignant tumors (Berthoin, Sahut-Barnola, Lambert-Langlais et al., 2010). Nearly all adrenocortical carcinomas (>90%) exhibit deregulated telomerase activity (Else, Giordano and Hammer, 2008). Mice carrying an inactivating mutation in *Tpp1/Acd*, which normally functions in the shelterin complex to protect telomeres, and a single wild-type *p53* allele develop diverse tumor types, including adrenocortical carcinoma at low frequency (Else, Trovato, Kim et al., 2009). These interesting findings in engineered mice raise the possibility that telomere defects may also contribute to adrenal tumor susceptibility in individuals with LFS. In support of this possibility, the age of tumor onset correlates with telomere length in LFS patients with germline *p53* mutations (Tabori et al., 2007). It has long been recognized that gonadectomy induces adrenocortical carcinoma in several specific mouse strains, but not others (e.g., C57BL/6J). Deletion of the TGF- β family member, *Inhibin- α* , cooperates with castration and ovariectomy and nearly all male and female mice develop adrenocortical tumors, indicating that inhibin functions as a tumor suppressor in the adrenal cortex (Matzuk, Finegold, Mather et al., 1994). In support of these findings, *Inhibin- α* mutations and loss of expression has been observed in a subset of human adrenocortical carcinomas (Longui, Lemos-Marini, Figueiredo et al., 2004, Munro, Kennedy and McNicol, 1999).

As we learn more about the biology that controls the normal development of the adrenal cortex and the factors that drive tumor initiation and progression of this gland, genetically-engineered mice will become more integral for establishing the relevancy of these findings. Most importantly, the development of animal models that can phenocopy adrenocortical carcinoma, including the establishment of human tumor xenografts in mice, will provide the

opportunity to test new chemotherapeutic agents that will hopefully lead to improved long-term outcome of patients with ACC.

10. Therapeutic targeting of p53 pathway

In the setting of a well-established role for compromised p53 signaling in a large proportion of ACCs, particularly those in children, reconstitution of a functional p53-pathway would seem an attractive focus for rationally-designed therapeutics. Unfortunately, despite the wealth of research aimed at augmenting p53 signaling in cancers, the pathway has thus far remained an elusive therapeutic target. Nonetheless, recent advances lend promise to the possibility of therapeutic manipulation of the pathway in tumors with aberrant p53-signaling (Martins, Brown-Swigart and Evan, 2006, Ventura, Kirsch, McLaughlin et al., 2007, Xue, Zender, Miething et al., 2007).

Of prime concern in systemically targeting p53 itself is the potential effect of any manipulation of p53 in untransformed healthy cells, wherein p53 plays an integral role in normal cell physiology. Among the more promising strategies under investigation is the targeting of regulators of p53, rather than the p53 molecule itself. As previously mentioned, MDM2 and MDM4/MDMX form heterodimers which bind p53 and target it for destruction via polyubiquitination. Mutations that interrupt transcriptional activity of p53 thus lead to lower levels of MDM2 transcript and result in accumulation of mutant p53 protein. Small molecule inhibitors of the interactions between the MDM2/MDM4 complex and p53 such as the nutlins (Ding, Lu, Nikolovska-Coleska et al., 2006, Grasberger, Lu, Schubert et al., 2005, Vassilev, 2004), or those that interfere with the E3-ubiquitin ligase activity of MDM2 (Yang, Ludwig, Jensen et al., 2005) have been shown to stabilize p53 and restore tumor-suppressive activity and cause tumor regression in xenografts (Klein and Vassilev, 2004, Vassilev, 2004, Vassilev, Vu, Graves et al., 2004), however, their effects on normal tissues are still unclear. Moreover, while this strategy is promising, it is also self-limiting, since MDM2 is a transcriptional target of p53, and thus restoration of p53 activity also ultimately stands to augment MDM2 expression, thus negating its inhibition. MDM4/MDMX expression is not under the control of p53, and may thus be a more appropriate target for inhibition. Indeed a small-molecule inhibitor of MDMX was recently described (Popowicz, Czarna, Wolf et al., 2010, Reed, Shen, Shelat et al., 2010). Additionally, histone deacetylase inhibitors have been shown to induce downregulation of both MDM2 and MDM4 (Palani, Beck and Sonnemann, 2010).

An unexpected result of a large cell-based reporter assay screen of natural compounds, was the observation that low-dose Actinomycin D resulted in a specific induction of p53 activity (Choong, Yang, Lee et al., 2009). In addition, expression array analyses of the effects of Actinomycin D were indistinguishable from those of the MDM2 inhibitor nutlin in p53 wild-type and mutant cells. While the specific mechanism of action for Actinomycin D in regulating p53 activity is still unclear, it may turn out to be a valuable tool in combination therapy for p53-dependent tumors (Rao, van Leeuwen, Higgins et al., 2010).

A further mode of downregulation of p53 activity occurs via deacetylation of lysine 382 in the C-terminus of the protein. This is accomplished, in part, by the actions of the SIRT1 and SIRT2 deacetylases. Consequently, inhibition of SIRT1 and SIRT2 stands to block deacetylation of p53 and increase p53-activity. A class of small-molecule inhibitors of SIRT1 and SIRT2, called Tenovins, were recently identified (Lain, Hollick, Campbell et al., 2008, van Leeuwen, Higgins, Campbell et al., 2011). While clinical efficacy remains to be demonstrated, Tenovins might ultimately function to stabilize p53, allowing protection of the genome and facilitating elimination of genetically corrupted cells via apoptosis.

An alternative strategy to interfering with MDM2/MDM4/p53 interactions, involves screening large libraries for agents that interact selectively with mutant forms of *p53*. Indeed, the recognition that a majority of tumors express an aberrant *p53* molecule, lends theoretical credence to this approach. Whether it is achievable, however, is a different question. Promising initial results were reported with CP-31398, a small molecule identified in a *p53* stabilization assay-based screen (Foster, Coffey, Morin et al., 1999); however, the mechanism of action was subsequently shown to be non-specific to *p53*, but rather interacts directly with DNA (Rippin, Bykov, Freund et al., 2002). Nevertheless, this compound may have therapeutic potential in combination with other *p53*-reactivating compounds and/or chemotherapy (Huang, Zhang, Tavaluc et al., 2009, Rao, Steele, Swamy et al., 2009, Roh, Kang, Minn et al., 2011). Large-scale screens of combinatorial small-molecule libraries using functional selection for selective restoration of wild-type *p53* activity may provide further leads in this route of investigation.

Recent identification of a small molecule that binds to the DNA-binding domain of *p53* and stabilizes interactions with DNA was identified and shown to reactivate DNA binding *p53* mutant R273H and structural mutant R249S (Demma, Maxwell, Ramos et al., 2010). This compound was also demonstrated to interrupt MDM2-mediated ubiquitination. Treatment of tumor cells and xenografts harboring *p53*-mutations with this compound resulted in the induction of apoptosis and reduction of tumor growth. Knowing that the vast majority of pathogenic mutations in *p53* lie within the DBD, which forms an inherently unstable interaction with DNA that is further destabilized by mutation, approaches such as this to stabilize the interaction hold potential, although much pharmacologic characterization and optimization remains before this becomes a realistic approach.

Finally, the unique biochemical characteristics of the R337H mutant and its pH-dependent loss-of-function may offer a distinct mechanism of *p53*-restoration, by targeting cellular mechanisms that regulate intracellular proton transport, such as the sodium-hydrogen exchanger family of cell-surface proteins, inhibitors of which currently exist in clinical practice (Masereel, Pochet and Laeckmann, 2003, Tracey, Allen, Frazier et al., 2003).

It is quite likely that a “one-size-fits-all” approach to targeted therapies based on mutant *p53* may not be achievable, given the different effects individual mutants will have on the protein, whether by affecting conformation, DNA binding or regulation. Unlike several of the recent success stories with targeted therapeutics in chronic myelogenous leukemia and melanoma, wherein a single genetic aberration could be targeted in a large proportion of disease carriers, the multitude of mutations in *p53* may limit development of precise targeted therapeutics, or may require the adoption of mutant-specific reactivating drugs (as has recently been demonstrated for the Y220C mutant (Boeckler, Joerger, Jaggi et al., 2008)). In addition, the sheer challenge of activating a functionally inactivated (or disrupted or even completely absent) tumor suppressor is much more daunting than identifying agents that might target and ‘turn off’ an activated oncogene pathway.

It has become clear, in the more than 20 years since the identification of ACC as a component of the LFS, that *p53* plays a dominant role in the pathogenesis of pediatric ACCs, while its role in adult tumors remains to be defined. A spectrum of mutations with varying functional consequence illustrates the unique response of the adrenal gland to *p53* loss and distinguishes this tissue from others affected by LFS. The response of the adrenal cortex to *p53* deficiency illustrates potential mechanisms for acquisition of the transformed phenotype. Finally, therapeutic alternatives to the current standard-of-care for ACC may offer hope for reduced toxicity and improved outcomes.

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Highlights

- Adrenocortical carcinomas present in excess among carriers of germline *p53* mutations
- *p53*-associated ACC occurs predominantly in the pediatric age group
- Mouse models of *p53*-loss do not develop ACC
- Pediatric ACC is occasionally associated with low-penetrance *p53* alleles which retain substantial protein activity
- Elements of adrenal development, including regression of the fetal zone, may explain a unique predisposition to tumorigenesis in children.

Figure 1.

The primary structure of p53 identifies multiple functional domains including a transactivation domain (TAD), proline-rich region (PRR), DNA-binding domain (DBD), tetramerization domain (TET) and regulatory region (REG). Codon positions are indicated by the numbers below the figure. The common “hotspot mutations” at codons 175, 245, 248, 249, 273 and 282 all lie within the DBD.

A. **B.** **Figure 2.**

A. Data from the Surveillance Epidemiology and End Results registry recapitulate the bimodal distribution that has been previously described, with peaks in early childhood and middle-age (SEER Data, 1973-2008).

B. Age of diagnosis of ACC among *p53*-germline mutation carriers. A unimodal distribution is observed with 54% of patients, presenting before age 2 and 92% prior to age 18. These data include 76 individuals with the R337H allele. A similar distribution is observed when excluding these individuals from the analysis (not shown). Data from IARC Database, R. 15(Petitjean et al., 2007).

Figure 3. Codon distribution of p53 missense germline mutations in all individuals reported in IARC database (above) vs. in patients with ACC (below). The R337H mutation (arrows) is the most frequently reported mutation in both groups.