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ORIGINAL REPORT

Identification of Clinical and Biologic Correlates Associated With Outcome in Children With Adrenocortical Tumors Without Germline *TP53* Mutations: A St Jude Adrenocortical Tumor Registry and Children's Oncology Group Study

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ABSTRACT

Purpose
The clinical features, pathogenesis, and outcomes in children with adrenocortical tumors (ACTs) without germline *TP53* mutations have not been systematically studied. Herein, we describe these correlates and analyze their association with outcome.

Patients and Methods
Genomic DNA was analyzed for *TP53*, *CTNNB1*, *CDKN1C*, *ATRX*, and chromosome 11p15 abnormalities. β-catenin expression and Ki-67 labeling index (LI) were evaluated by immunostaining. Primary end points were progression-free (PFS) and overall survival.

Results
Median age of 42 girls and 18 boys was 3.3 years (range, 0.25 to 21.7 years). Complete resection (stages I and II) was achieved in 32 patients, and 28 patients had stage III or IV disease. Constitutional abnormalities of chromosome 11p15 occurred in nine of 40 patients, with six patients not showing phenotype of Beckwith-Wiedemann syndrome. Three-year PFS and overall survival for all patients were 71.4% and 80.5%, respectively. In single-predictor Cox regression analysis, age, disease stage, tumor weight, somatic *TP53* mutations, and Ki-67 LI were associated with prognosis. Ki-67 LI and age remained significantly associated with PFS after adjusting for stage and tumor weight. Three-year PFS for 27 patients with Ki-67 LI $\geq$ 15% was 48.5% compared with 96.2% for 29 patients with Ki-67 LI $<$ 15% (log-rank $P = .002$), and the rate of relapse increased by 24% with each 1-year increase in age at diagnosis (hazard ratio, 1.24; $P = .0057$).

Conclusion
Clinicopathologic features and outcomes of children with ACTs without germline *TP53* mutations overlapped those reported for children with germline *TP53* mutations. Our findings highlight the central role of genetic or epigenetic alterations on chromosome 11p15 in pediatric ACTs. Ki-67 LI is a strong prognostic indicator and should be investigated to improve the histologic classification of pediatric ACTs.

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INTRODUCTION

Pediatric adrenocortical tumor (ACT) is an uncommon malignancy frequently associated with Li-Fraumeni syndrome, a familial cancer predisposition disorder caused by germline mutations in the tumor suppressor gene *TP53*.[1,2] ACT is rarely associated with other genetic constitutional disorders such as Beckwith-Wiedemann syndrome (BWS), which results from deregulation of a gene cluster on chromosome 11p15[3]; multiple endocrine neoplasia; neurofibromatosis; familial adenomatous polyposis; or congenital adrenal hyperplasia.[4,5]

Our recent study[6] showed that copy-neutral loss of heterozygosity (LOH) of chromosomes 11 and 17 is the hallmark of pediatric ACTs associated with germline *TP53* mutations (mut*TP53*-ACTs). Combined loss of maternal chromosome 11, duplication of paternal chromosome 11, and loss of chromosome 17 harboring wild-type *TP53*

ASSOCIATED CONTENT

Appendix
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occurs early in pediatric adrenocortical tumorigenesis. In general, as the tumor increases in weight, it acquires additional genetic alterations and exhibits increasingly aggressive behavior.[6]

However, approximately 50% of children with ACTs do not harbor germline *TP53* mutations.[7] The mechanisms of tumorigenesis and tumor progression as well as prognostic markers of ACT in these patients have not been systematically evaluated. In this retrospective study, we report clinical features, molecular attributes, and outcomes in a relatively large cohort of children with ACTs without germline *TP53* mutations (wt*TP53*-ACTs).

PATIENTS AND METHODS

Patients and Biologic Samples

This was a retrospective study of patients with ACTs carrying germline wild-type *TP53* who were age < 22 years and enrolled in the St Jude Children's Research Hospital International Pediatric Adrenocortical Tumor Registry (ClinicalTrials.gov identifier: NCT00700414; n = 48) between 2003 and 2015 or the Children's Oncology Group (COG) ARAR0332 study (ClinicalTrials.gov identifier: NCT00304070; n = 12) between 2006 and 2013. Matched blood and tumor DNA was collected from 40 patients. For 20 patients, only tumor DNA (n = 14) or blood DNA (n = 6) was available for molecular studies.

Written informed consent was obtained from parents or legal guardians. The study was approved by the St Jude Institutional Review Board and the COG Rare Tumors Committee. Disease was staged according to reported guidelines.[8] Clinical management was uniform for those enrolled in COG ARAR0332 and varied as directed by primary physicians for those enrolled in the International Pediatric Adrenocortical Tumor Registry.

TP53, CDKN1C, ATRX, and CTNNB1 Mutational Status

TP53 and *CDKN1C* coding regions, including the flanking intronic sequence of each exon, were amplified by polymerase chain reaction and sequenced on a 3730xl DNA Analyzer (Applied Biosystems). Large deletions of *TP53* and *ATRX* were assessed using commercial multiplex ligation-dependent probe amplification (MLPA) kits (PO56 *TP53* probemix and P013 *ATRX* probemix; MRC-Holland, Amsterdam, the Netherlands) as per manufacturer instructions.

Mutational status of *CTNNB1* exon 3 was determined as previously described.[6] Immunostaining for β-catenin was performed on 4-μm formalin-fixed paraffin-embedded tumor sections. Monoclonal Beta-Catenin (14; Ventana Medical Systems, Tucson, AZ) was used as the primary antibody and visualized using the iVIEW DAB Detection Kit (Ventana Medical Systems).

Chromosome 11p15 Abnormalities

Matched blood and tumor DNA from 40 patients was analyzed for 11p15 chromosomal copy number alterations by using microsatellite analysis.[6] Gene dosage and methylation status were evaluated in blood samples by using ME030-C3 BWS/RSS probemix (MRC-Holland) as previously described[9] and quantified using Coffalyzer software (MRC-Holland).

Ki-67 Immunostaining

Formalin-fixed paraffin-embedded tumor sections were analyzed for Ki-67 nuclear expression using primary polyclonal rabbit antisera for Ki-67 (dilution, 1:500; Novocastra; Leica Biosystems, Buffalo Grove, IL) and stained using routine protocols. Ten tumor areas were examined under ×400 magnification, and the overall percentage of cells with Ki-67–positive staining among the 10 fields was scored as the Ki-67 labeling index (LI). Ki-67 LI was considered high if ≥ 15% of tumor cell nuclei were stained.

Gene Expression Analysis

PTTG1, *BUB1B*, *AURKB*, *HLA-DPA1*, and *MKI67* mRNA expression data were obtained from the Gene Expression Omnibus (GEO) repository including 63 pediatric ACTs (GEO databases GSE76019 and GSE76021). Experiments were performed as previously described.[10]

Statistical Analyses

The Kaplan-Meier method was used to estimate progression-free (PFS) and overall survival (OS). PFS was defined as time elapsed from diagnosis to progression or death, with times censored at the date of last follow-up. OS was defined as time elapsed from diagnosis to death, with times for living patients censored at last follow-up. The log-log method was used to calculate the CIs for survival estimates.[11,12] The log-rank test was used to compare PFS or OS across groups according to presence or absence of molecular and clinical features. Cox proportional hazards regression models were used to evaluate the association of clinical and molecular features with PFS or OS while accounting for stage (dichotomized as stage ≤ IV). The Wilcoxon rank sum test was used to explore Ki-67 LI status in relation to age and tumor weight. The Wilcoxon rank sum test was also used to determine association between mRNA gene expression and *TP53* status, and Cox regression was used to determine association between gene expression and PFS. All tests were two sided, and no multiple-testing adjustments were performed.

RESULTS

Patient Demographics and Clinical Correlates

Median age of the 60 eligible patients (42 females and 18 males) was 3.3 years (range, 0.25 to 21.7 years). Signs and symptoms of virilization alone and associated with hypercortisolism (Cushing syndrome) were seen in 23 and 10 patients, respectively. There were no endocrine clinical signs or symptoms in 12 patients. Tumors were histologically characterized as carcinoma (n = 43), adenoma (n = 10), and undetermined (n = 7). Thirty-two patients had localized disease (stage I or II), and 28 had advanced disease (stage III or IV). Clinical data are summarized in Table 1 and detailed in Appendix Table A1 (online only).

TP53, CTNNB1, and ATRX Mutational Status in Tumor Samples

TP53 was analyzed in 54 of 60 patients, and somatic *TP53* alterations were observed in nine samples (Fig 1A), including six missense mutations (R158H, R175H, E180K, M246V, R273C, and R273H), one nonsense mutation in exon 10 (R342*), and one frameshift insertion at exon 5 (c.403_404insT).[13] Complete deletion of *TP53* occurred in one sample.

Activation of the Wnt pathway was determined by Sanger sequencing of *CTNNB1* exon 3 (n = 48) and by β-catenin immunostaining (n = 5). Mutations (n = 22) or positive nuclear expression of β-catenin (n = 1) were identified in 23 (43%) of the 53 samples (Figs 2A and 2B). In four of these samples, mutations were acquired in both *CTNNB1* and *TP53* (WT006, WT007, WT008, and WT050; Appendix Table A1).

ATRX intragenic deletions were detected in two of 53 ACTs analyzed by MLPA: one sample showed a deletion of exons 11 to 30 in the primary tumor and lung metastasis (WT006), whereas the other showed a deletion in exons 6 to 15 (WT010). A missense *ATRX* mutation (R2164S) was also identified in one of 12 samples

Table 1. Baseline Patient Demographic and Clinical Characteristics

Characteristic	No. (%)
Sex	
Female	42 (70)
Male	18 (30)
Age at presentation, years	
Median	3.3
Range	0.25-21.7
Endocrine signs	
Virilization alone	23 (38)
Virilization plus cushing	10 (17)
Cushing alone	12 (20)
No clinical signs	12 (20)
Aldosterone-producing tumor	3 (5)
Tumor histology	
Adenoma	10 (17)
Carcinoma	43 (72)
Undetermined	7 (12)
Ki-67 LI ≥ 15%	27 (48) of 56
Surgery	
Complete resection	32 (53)
Microscopic residual disease	28 (47)
Tumor spillage	10
Disease stage	
I	18 (30)
II	14 (23)
III	13 (22)
IV	15 (25)
Tumor weight, g	
< 200	39 (68) of 57
≥ 200	18 (32) of 57
Treatment	
Surgery alone	36 (60)
Surgery followed by chemotherapy	23 (38)
Chemotherapy alone	1 (2)
Outcome	
Alive and free of disease	46 (77)
Alive with disease	1 (1)
Died	13 (22)
Molecular markers	
Somatic 11p15 LOH	31 (86) of 36
Somatic *CTNNB1* mutations/nuclear expression	23 (43) of 53
Somatic *TP53* mutations	9 (17) of 54
Somatic *ATRX* mutations	3 (6) of 53

Abbreviations: LI, labeling index; LOH, loss of heterozygosity.

analyzed by whole-exome sequencing (WT009).[6] All three tumor samples with an *ATRX* mutation also harbored a somatic *TP53* mutation (Appendix Table A1).

Germline and Somatic 11p15 Genetic and Epigenetic Alterations

Chromosome 11p15 encodes a cluster of imprinted genes that positively and negatively regulate cell proliferation and survival.[14-16] These genes are differentially expressed in a parental origin–dependent manner through H19DMR (imprinting control region 1 [IC1]) and KvDMR (IC2). Insulin-like growth factor 2 (IGF2) is selectively expressed from the paternal allele, whereas the cell-cycle inhibitor CDKN1C ($p57^{KIP2}$) is preferentially expressed from the maternal allele. High expression of IGF2 and low expression of CDKN1C, as seen in most pediatric ACTs,[6] likely result from copy-neutral LOH or imprinting defects within the 11p15 locus.[6,17]

Microsatellite markers and methylation-specific MLPA analysis for chromosome 11p15 in blood DNA of 40 patients revealed a homozygous pattern for all markers and hypermethylation at IC1 and hypomethylation at IC2 (Appendix Fig A1A, online only), indicative of paternal uniparental disomy (UPD), in four patients (WT001, WT031, WT043, and WT049), two of whom did not exhibit clinical signs of BWS (WT0031 and WT043). In another patient (WT002), a mosaic paternal UPD for 11p15 was identified, with partial gain of methylation at IC1 and partial loss of methylation at IC2 (Appendix Fig A1B). An additional four patients (WT003, WT009, WT023, and WT030) exhibited isolated loss of methylation at IC2 (Appendix Fig A1C), suggesting silencing of maternal alleles, as observed in 50% of patients with BWS.[18] Of note, patient WT003 had an incomplete BWS phenotype (hemihypertrophy), but no other features of BWS were seen in the remaining three patients. *CDKN1C* mutations were not detected in the germline DNA from 46 patients. Finally, analysis of corresponding tumors revealed somatic LOH in 31 (86%) of 36 informative patient cases, with selective loss of the maternal chromosome in all patients for whom parental blood DNA was available (n = 11; Appendix Fig A1D). In addition, microsatellite analysis of 14 patients for whom only tumor DNA was available, revealed a homozygous pattern suggestive of LOH in 12 (86%), and UPD was excluded in all patients for whom only blood DNA was available (n = 6).

Ki-67 LI

Immunohistochemical analysis of Ki-67 was performed in tumor samples from 56 patients. Ki-67 LI was positive (≥ 15% tumor-cell nuclei stained) in 27 patients (48%; Fig 3A; Appendix Table A1).

Outcome, Treatment, and Prognostic Factors

Of 60 patients, 32 were considered free of disease after surgery, 13 had microscopic residual disease or tumor spillage during surgery (stage III), and 15 had overt metastatic disease (stage IV). The primary tumor was surgically resected in all cases except in patient WT056. No patients with stage I (n = 18) or II (n = 14) disease (excluding WT017, WT019, and WT027) received adjuvant chemotherapy. Patients with tumor spillage or microscopic residual disease were typically treated with platinum-based chemotherapy and mitotane. At a median follow-up of 3.4 years, 47 patients were alive, and 46 of them were free of disease. Thirteen patients died as a result of progressive disease. The 3-year PFS and OS for all patients were 71.4% (95% CI, 60.3% to 84.4%) and 80.5% (95% CI, 70.2% to 92.3%), respectively.

Consistent with earlier findings from adult ACTs,[19] the presence of somatic *TP53* mutations was significantly associated with poor prognosis. The 3-year PFS rates for patients with or without somatic *TP53* mutations were 38.9% (95% CI, 9.3% to 68.7%) and 79.4% (95% CI, 64.0% to 88.7%; $P = .02$), respectively (Fig 1B). When stage IV disease was included in the Cox regression model, presence of somatic *TP53* mutations was no longer associated with outcome ($P = .21$; Fig 1C). *CTNNB1* status was not significantly associated with PFS ($P = .80$; Fig 2C).

The 3-year PFS for the 27 patients with Ki-67 LI ≥ 15% was 48.5% (95% CI, 27.5% to 66.7%) compared with 96.2% (95% CI, 75.7% to 99.4%) for the 29 patients with Ki-67 LI < 15% ($P = .002$;

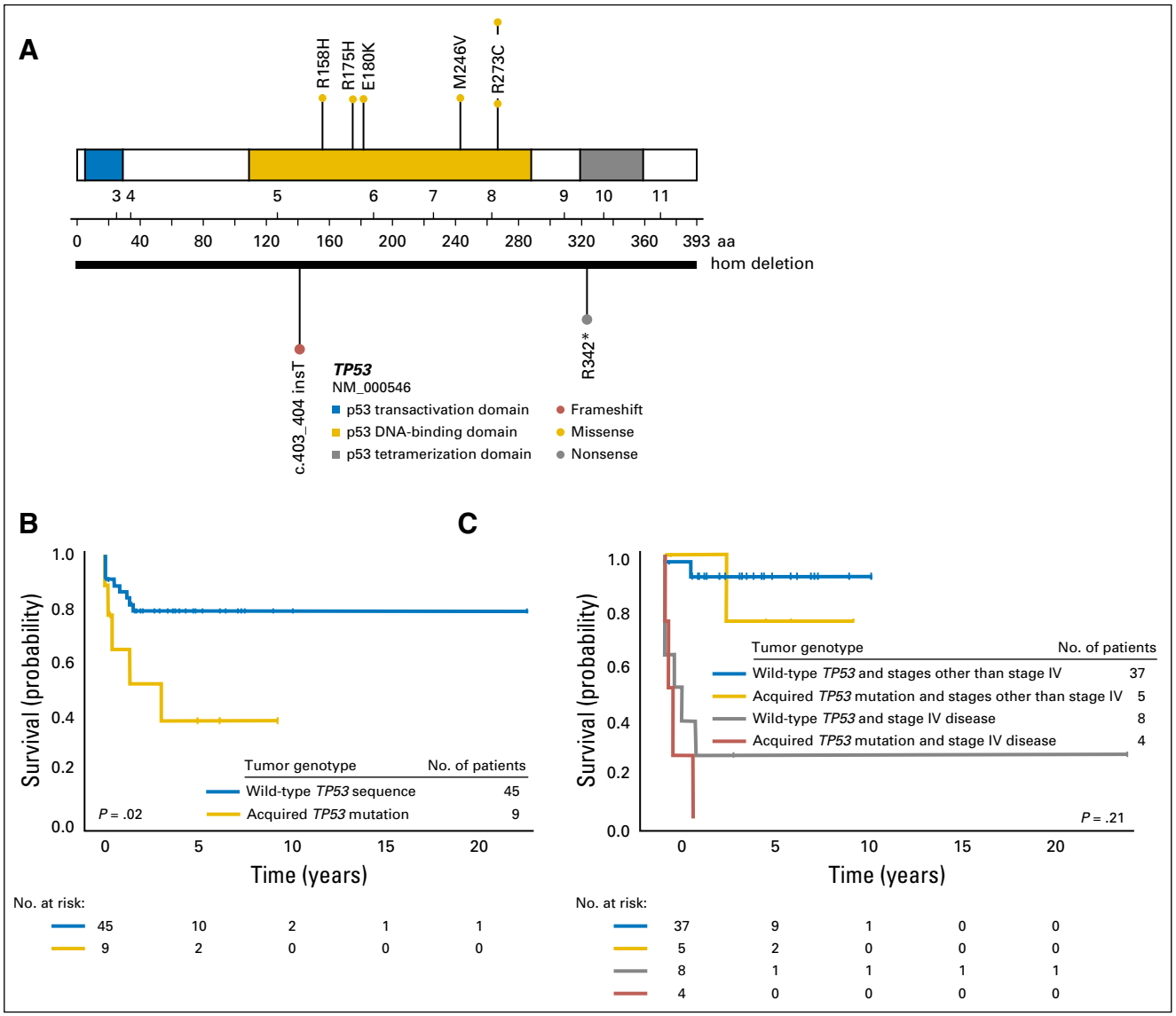

Fig 1. Somatic *TP53* mutations in patients with adrenocortical tumors (ACTs) without *TP53* mutations. (A) Schematic diagram of acquired *TP53* mutations. (B) Kaplan-Meier estimates for progression-free survival (B) in pediatric patients with ACTs with or without acquired *TP53* mutations and (C) by *TP53* status and disease stage. After discriminating for disease stage and including stage IV disease in the Cox regression model, presence of somatic *TP53* mutations was no longer associated with outcome ($P = .21$). hom, homozygous.

Fig 3B). Ki-67 LI remained significantly associated with PFS after adjusting for disease stage ($P = .0048$; Fig 3C). Cox regression modeling showed that among these 56 patients, rate of progression or death for patients with stage IV disease was 21.9 times that for other patients (95% CI, 5.25 to 91.46; $P < .001$), and rate of progression or death for patients with Ki-67 LI $\geq$ 15% was 22.27 times that for patients with Ki-67 LI $<$ 15% (95% CI, 2.57 to 192.53; $P = .0048$).

Because higher tumor weight and older age are associated with poor prognosis in children with ACTs in general,[8,20-22] we analyzed their association with Ki-67 LI. Median tumor weight was significantly higher for patients with Ki-67 LI $\geq$ 15% than for those with Ki-67 LI $<$ 15% (191 *v* 93.2 g; $P = .02$). Ki-67 LI was not significantly associated with age ($P = .62$), suggesting that Ki-67 LI and age are independently associated with outcome. Thus, we fit a Cox regression model with age, tumor weight, disease stage (stage IV *v* I to III), and Ki-67 LI ($<$ 15% *v* $\geq$ 15%) as predictors of PFS. In this model, tumor weight and disease stage showed potentially meaningful associations with PFS but did not reach statistical significance. However, rate of progression or death for patients with Ki-67 LI $\geq$ 15% was 41.8 times that for patients with Ki-67 LI $<$ 15% (95% CI, 3.51 to 497.8; $P = .003$), and rate of relapse increased by 24% with each 1-year increase in age at diagnosis (hazard ratio, 1.24; 95% CI, 1.07 to 1.45; $P = .0057$; Appendix Table A2, online only).

We analyzed the expression of *MKI67*, which encodes Ki-67 protein, in two independent gene expression data sets from the GEO repository of pediatric ACTs and found that *MKI67* overexpression as a single predictor was associated with worse PFS in both cohorts (GSE76021, $P = .001$; GSE76019, $P = .016$) as well as after adjustment for stage IV disease (GSE76021, $P = .001$; GSE76019, $P = .011$).

Consistent with these findings, greater mRNA expression of *MKI67*, *PTTG1*, *BUB1B*, and *AURKB* was found in ACT with

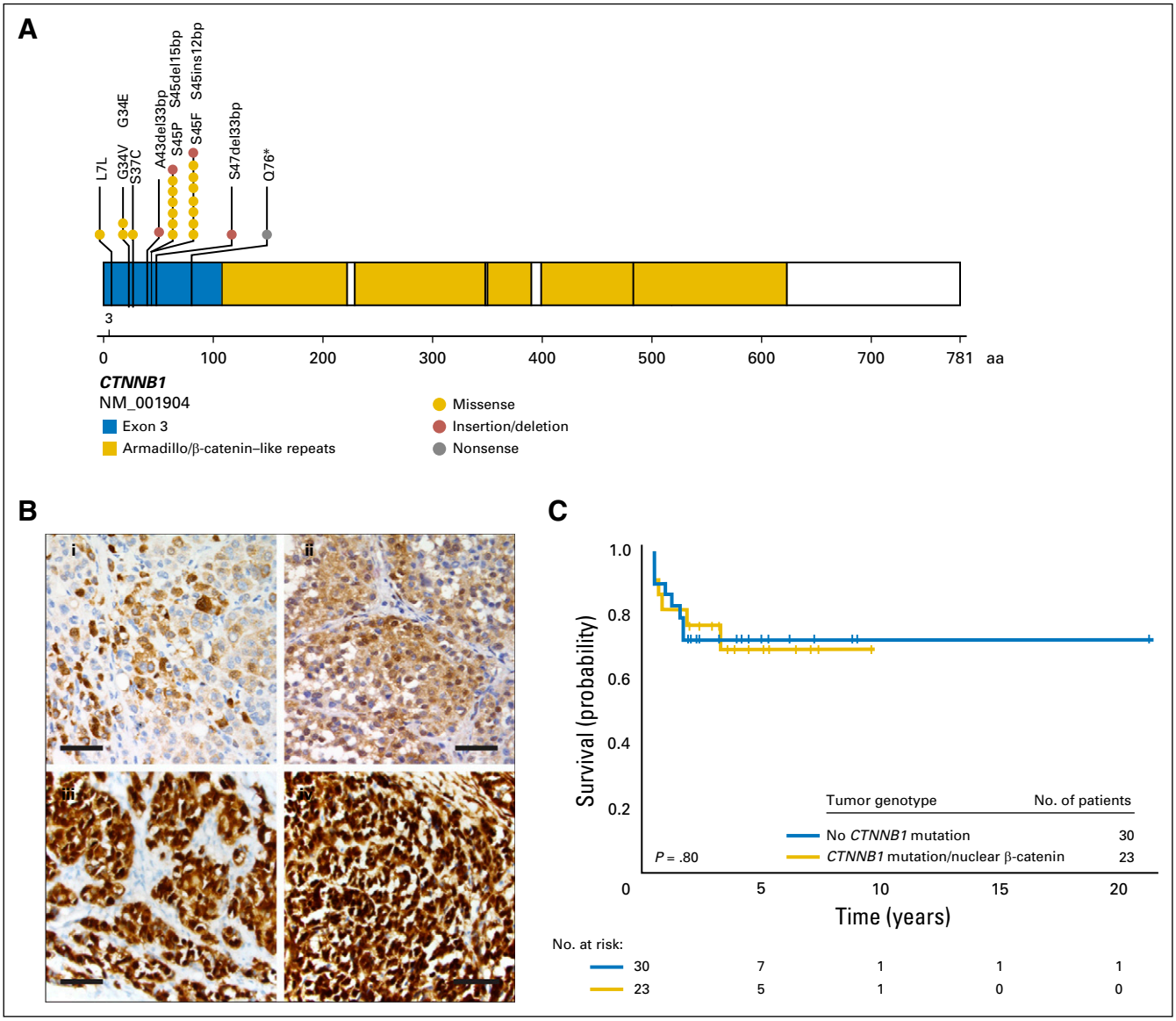

Fig 2. Somatic *CTNNB1* mutations in patients with adrenocortical tumors (ACTs) without *TP53* mutations. (A) Schematic diagram of activating *CTNNB1* mutations. (B) Immunohistochemical analysis of β-catenin and yes-associated protein 1 (YAP1) in pediatric ACTs. YAP serves as a surrogate marker for β-catenin expression. Moderate nuclear immunostaining of (i) β-catenin and (ii) YAP in a tumor with the S45P mutation in *CTNNB1*. Strong nuclear immunoreactivity for (iii) β-catenin and (iv) YAP in a pediatric patient with unknown *CTNNB1* molecular status. These results are indicative of Wnt signaling pathway activation. Size bars represent 50 μm. (C) Kaplan-Meier estimates for progression-free survival by *CTNNB1* status ($P = .80$).

adverse prognostic features (Appendix Fig A2, online only). Furthermore, *MKI67* overexpression was correlated with low levels of *HLA-DPA1* (Appendix Fig A3, online only), which is significantly associated with poor outcome.[10,23] Expression of *PTTG1*, *BUB1B*, and *AURKB* was significantly higher in those with mut*TP53*-ACTs compared with wt*TP53*-ACTs. However, expression of *IGF2* and *MKI67* was similar, irrespective of patients' *TP53* status (Appendix Fig A4, online only).

DISCUSSION

Our study reveals significant overlap in clinicopathologic features, molecular attributes, pathogenesis, and outcomes among children with wt*TP53*-ACTs and mut*TP53*-ACTs and captures differences between these groups (Appendix Table A3, online only). Approximately 90% of patients with mut*TP53*-ACTs develop adrenocortical tumors by 5 years of age (peak incidence, 1 to 3 years); thereafter, the risk decreases and remains low throughout life.[21,22] In contrast, 37% of our patients with wt*TP53*-ACTs were diagnosed after the age of 5 years. More than 90% of children with mut*TP53*-ACTs secrete androgens or androgens plus cortisol,[24] whereas this pattern of hormonal secretion is seen in only approximately 55% of patients with wt*TP53*-ACTs.

The differences in age of onset and hormonal secretion pattern by ACTs may be attributed to adrenal cortex cell types involved in the malignant transformation. Our findings suggest that in children age < 5 years, regardless of genetic background, ACTs arise from the transient embryonic adrenal cortex.[25,26] A smaller incidence peak in pubertal patients with wt*TP53*-ACTs coincides with physiologic changes in the adrenal cortex during

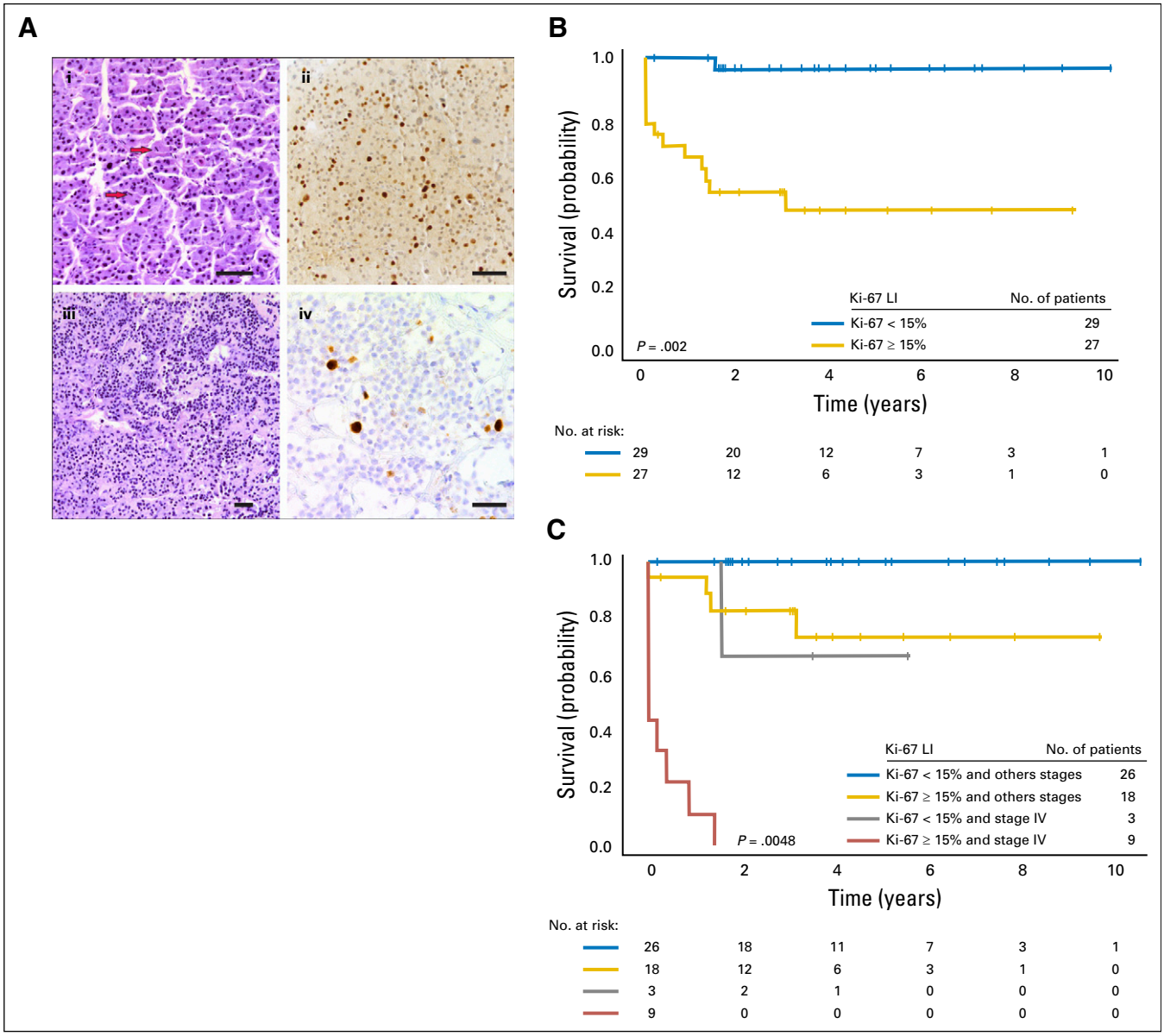

Fig 3. (A) Hematoxylin and eosin staining and Ki-67 labeling index (LI) in pediatric adrenocortical tumors without *TP53* mutations: (i, ii) tumor with a high (≥ 15%) Ki-67 labeling index (LI; red arrows indicate mitotic figures), (iii, iv) tumor with a low (< 5%) LI. Size bars represent 50 μm. (B) Kaplan-Meier estimates for progression-free survival (PFS) according to Ki-67 LI showing that Ki-67 LI < 15% was significantly associated with longer PFS ($P = .002$). (C) Kaplan-Meier estimates for PFS according to Ki-67 LI and disease stage. Ki-67 LI remained significantly associated with outcome after adjusting for disease stage ($P = .0048$).

adrenarche.[27] Strikingly, the female predominance in ACTs at a young age (3:1) was not observed in children age ≥ 10 years (1:1) in this study. Collectively, our findings and previous studies suggest that predisposition to pediatric ACT depends on constitutional factors such as *TP53* mutations and chromosome 11p15 abnormalities[2,28-31] and physiologic sex- and age-specific developmental changes in the adrenal cortex.[32]

Remarkable and consistent features of adrenocortical tumors include chromosome 11p15 abnormalities and *IGF2* overexpression,[6,33-36] which are early events in adrenal cortex tumorigenesis.[6] *IGF2* overexpression probably occurs as a result of the combination of loss of maternal chromosome 11 and duplication of paternal chromosome 11,[6,34] which occurs in virtually all patients, irrespective of *TP53* status.[6]

In this study, dysregulation of imprinted genes in chromosome 11p15 was noted in the germline of nine of 40 patients. Also, insertion of foreign viral DNA (human herpesvirus 6 [HHV-6]) in the telomeric region of the short arm of chromosome 11 occurred in two patients (WT005 and WT030), both of whom inherited integrated HHV-6 from their fathers.[6] HHV-6 chromosomal integration is associated with changes in the host methylation machinery to facilitate integration.[37] In fact, patient WT030 also had hypomethylation at IC2. These findings suggest that irrespective of chromosome 11p15 status in the germline, adrenocortical tumorigenesis requires *IGF2* overexpression and loss of a segment of or the entire maternal chromosome 11p15 (copy-neutral LOH). Our observations are consistent with the observation that in BWS, predisposition to ACT is highest among those with germline paternal UPD.[38]

Of clinical interest, six (67%) of nine patients in our study harbored germline abnormalities of chromosome 11p15 but did not have clinical signs of BWS. In these patients, *IGF2* overexpression (Appendix Fig A1E) might have been restricted to tissues in which chromosome 11 underwent somatic copy-neutral LOH. Consistent with this hypothesis, ACT tissues from all five patients with germline chromosome 11p15 abnormalities had LOH of chromosome 11p15 (Appendix Table A4, online only). Our findings underscore the importance of detailed molecular analysis of chromosome 11p15 status in the germline of all children with embryonic tumors, especially Wilms tumor, hepatoblastoma, rhabdomyosarcoma, ACT, and pancreatoblastoma, all of which are associated with chromosome 11p15 abnormalities.[39] Approximately 90% of adult adrenocortical carcinomas also overexpress *IGF2*.[17,30] Attempts to pharmacologically target the IGF2 pathway with the IGF1 receptor inhibitor linsitinib in adult ACT was not associated with improved outcome.[40] This strategy has not been used in pediatric ACT.

Activation of the Wnt signaling pathway is common in adult adrenocortical adenomas and carcinomas,[41-44] suggesting its occurrence early in tumor formation.[6,43] In our patients with wt*TP53*-ACTs, activating mutations in *CTNNB1* were frequent and also observed in both adenomas and carcinomas. In contrast, activating mutations in *CTNNB1* are rare in patients with germline *TP53* mutations and seem to be mutually exclusive.[6]

Histologic classification of pediatric ACT has been controversial because of the lack of prognostic specificity.[20,45] Because clinical presentation (age of onset and tumor pattern of hormonal secretion) and molecular (germline *TP53* mutations, chromosome 11p15 abnormalities, and overexpression of *IGF2*) findings for pediatric ACT are similar to those for adenoma, carcinoma, and undetermined histology, we believe that they all represent a spectrum of the same disease.

A strong association between Ki-67 LI and prognosis in adult ACT has recently been established.[46] Ki-67 is a cell proliferation marker that is critical for chromosome segregation during cell division.[47] Given these findings, we investigated the prognostic implications of Ki-67 LI in our pediatric cohort. Ki-67 LI $\geq$ 15% and age were each independently associated with poor prognosis. To further explore Ki-67 prognostic correlates, we reanalyzed public data from our recent studies showing that expression of cell-cycle regulators and HLA class II genes was associated with outcome in pediatric ACT.[6,10] When *MKI67* mRNA expression was included in the analysis, it was positively associated with expression of cell-cycle regulatory genes and strongly associated with outcome. Therefore, Ki-67 LI may serve as an additional prognostic marker that should be considered in the current histopathologic classification of pediatric ACT.

In summary, our study reveals that clinical features, prognostic factors, and outcomes of pediatric ACT with or without germline *TP53* mutations are overlapping. However, some genetic alterations contributing to tumorigenesis are likely diverse between both groups, with *CTNNB1* mutations occurring almost exclusively in patients without germline *TP53* mutations. On the basis of this study and other previous findings, constitutional 11p15 abnormalities should be considered in all children with ACTs irrespective of the absence of features of BWS or other growth disorders. We also recommend that Ki-67 LI be included in histologic characterization to improve the pathologic classification of pediatric ACT.

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

Disclosures provided by the authors are available with this article at jco.org.

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Identification of Clinical and Biologic Correlates Associated With Outcome in Children With Adrenocortical Tumors Without Germline *TP53* Mutations: A St Jude Adrenocortical Tumor Registry and Children's Oncology Group Study

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Appendix

Table A1. Characteristics of Patients With ACTs

Patient	Sex	Endocrine Signs	Tumor Histology	Age at Diagnosis (years)	Tumor Stage	Tumor Weight (g)	Initial Treatment	Relapse	Died	*TP53* Mutations	*CTNNB1* Mutations	11p15 Status		Ki-67 ≥ 15%	Follow-Up Time (years)
												Blood	Tumor		
WT001	Female	V	ACA	8.8	I	21.5	S	No	No	Neg	Neg	UPD	ND	Neg	9.0
WT002	Female	C	ACC	0.5	II	148	S	No	No	Neg	Pos	UPD mosaic	LOH	Pos	2.9
WT003	Male	None	ACC	2.3	IV	56	S	No	No	Neg	Neg	Hypom IC2	LOH	Neg	5.3
WT004	Male	V + C	ACC	1.2	I	22	S	No	No	Pos	Neg	NA	LOH	Pos	9.2
WT005	Male	V + C	ACC	2.0	I	53	S	No	No	Pos	Neg	N	LOH	Pos	6.1
WT006	Female	V + C	ACC	8.1	IV	117	S + CT	Yes	Yes	Pos*	Pos	N	Neg	Pos	2.9
WT007	Female	V	Und	7.6	I	92	S	No	No	Pos	Pos	N	LOH	Neg	4.9
WT008	Female	None	ACC	0.9	I	30	S	Yes	No	Pos	Pos	N	LOH	Pos	6.8
WT009	Male	C	ACC	12.9	IV	304	S + CT	Yes	Yes	Pos*	Neg	Hypom IC2	LOH	Pos	3.7
WT010	Male	A	ACC	21.7	III	23	S + CT	No	No	Pos*	Neg	N	LOH	Pos	0.3
WT011	Male	None	ACC	17.1	IV	625	S + CT	No	Yes	Pos	Neg	N	Neg	Pos	0.2
WT012	Male	C	ACC	0.4	II	180	S	No	No	NA	NA	N	NA	Pos	3.4
WT013	Female	V	ACA	1.9	I	69.3	S	No	No	NA	NA	N	NA	Neg	8.2
WT014	Female	V + C	ACC	15.1	IV	592	S + CT	Persistent disease	Yes	NA	NA	N	NA	Pos	2.7
WT015	Female	C	ACC	9.0	IV	744	S + CT	No	Yes	NA	NA	N	NA	NA	4.1
WT016	Male	C	ACC	2.4	II	183.5	S	No	No	NA	NA	N	NA	Pos	3.0
WT017	Female	C	ACA	4.2	I	169	S + CT	No	No	Neg	Pos	NA	LOH	Neg	10.0
WT018	Female	V	ACC	3.9	I	5	S	No	No	Neg	Pos	NA	Neg	Neg	0.0‡
WT019	Male	V + C	ACC	3.3	II	144	S + CT	No	No	Neg	Pos	NA	LOH	Neg	0.2
WT020	Female	V	ACC	1.4	III	280	S	No	No	Neg	Neg	N	LOH	Pos	5.2
WT021	Female	None	ACC	13.2	IV	2,180	S + CT	No	Yes	Neg	Pos	N	LOH	Pos	1.3
WT022	Male	V	ACA	2.9	I	27	S	No	No	Neg	Neg	N	LOH	Neg	6.1
WT023	Female	V + C	Und	0.5	II	NA	S	No	No	Neg	Neg	Hypom IC2	LOH	Pos	3.7
WT024	Female	V	ACC	2.9	I	160	S	No	No	Neg	Neg	N	LOH	Neg	4.8
WT025	Female	C	Und	0.8	II	138	S	No	No	Neg	Neg	N	LOH	Neg	3.7
WT026	Female	None	ACC	1.1	II	172	S	No	No	Neg	Pos†	N	LOH	Pos	5.2
WT027	Female	V	ACC	8.9	II	239	S + CT	Yes	Yes	Neg	Neg	N	LOH	Pos	3.5
WT028	Female	V	Und	2.1	III	69	S	No	No	Neg	Neg	N	LOH	Neg	1.7
WT029	Female	V	ACA	4.9	I	21	S	No	No	Neg	Neg	N	LOH	Neg	7.2
WT030	Female	V	Und	1.3	I	28.4	S	No	No	Neg	Pos	Hypom IC2	LOH	Pos	7.4
WT031	Female	A	Und	11.6	III	388	S	No	No	Neg	Pos	UPD	ND	Neg	6.4
WT032	Female	None	ACC	7.3	III	60	S + CT	Yes	No	Neg	Neg	N	Neg	Pos	6.2
WT033	Female	None	ACC	15.0	II	192	S	Persistent disease	Yes	Neg	Neg	N	LOH	Pos	3.6
WT034	Male	V	ACC	13.3	IV	578	S + CT	No	Yes	Neg	Pos	N	LOH	Pos	1.5
WT035	Female	C	ACA	0.3	II	172	S	No	No	Neg	Pos	N	LOH	Neg	3.6
WT036	Female	C	ACC	12.3	IV	2,092	S + CT	No	Yes	Neg	Neg	N	Neg	Pos	0.3
WT037	Female	None	ACC	4.5	III	515	S	No	No	Neg	Neg	N	LOH	Neg	1.7
WT038	Male	V	ACA	1.2	I	62	S	No	No	Neg	Neg	N	LOH	Neg	1.3
WT039	Male	V	ACC	7.7	III	250	S + CT	No	No	Neg	Neg	N	LOH	Pos	2.9
WT040	Female	V	Und	14.6	III	1,360	S + CT	No	No	Neg	Neg	N	LOH	Neg	4.8
WT041	Female	V	ACC	15.8	II	620	S	No	No	NA	NA	N	LOH	Neg	2.9
WT042	Female	C	ACA	2.3	III	100	S	No	No	Neg	Pos	N	LOH	Neg	1.6
WT043	Female	None	ACC	1.1	IV	130	S + CT	No	No	Neg	Neg	UPD	ND	NA	22.5
WT044	Female	V + C	ACC	1.8	I	93.2	S	No	No	Neg	Pos	N	LOH	Neg	2.9

(continued on following page)

Table A1. Characteristics of Patients With ACTs (continued)

Patient	Sex	Endocrine Signs	Tumor Histology	Age at Diagnosis (years)	Tumor Stage	Tumor Weight (g)	Initial Treatment	Relapse	Died	*TP53* Mutations	*CTNNB1* Mutations	11p15 Status		Ki-67 ≥ 15%	Follow-Up Time (years)
												Blood	Tumor		
WT045	Male	V	ACC	2.4	II	766	S	No	No	Neg	Neg	N	LOH	Pos	2.0
WT046	Female	None	ACA	2.8	I	5.59	S	No	No	Neg	Pos	N	LOH	Neg	2.6
WT047	Female	A	ACA	15.8	I	25.2	S	No	No	Neg	Pos	N	Neg	Neg	2.0
WT048	Female	V	ACC	1.3	III	NA	S	No	No	Neg	NA	N	NA	NA	4.7
WT049	Female	C	ACC	1.5	II	120	S	No	No	Neg	Pos	UPD	ND	Neg	4.3
WT050	Male	None	ACC	13.3	IV	579	S + CT	Yes	Yes	Pos	Pos	N	LOH	Pos	1.5
WT051	Male	V + C	ACC	3.3	III	144	S + CT	No	No	Neg	Pos	NA	LOH	Neg	7.1
WT052	Female	V + C	ACC	1.2	IV	80	S + CT	No	No	Neg	Pos	NA	LOH	Neg	3.4
WT053	Female	V	ACC	17.1	IV	1,000	S + CT	Yes	Yes	Neg	Pos	NA	Neg	Neg	2.4
WT054	Male	V + C	ACC	15.0	IV	579	S + CT	Yes	Yes	Neg	Neg	NA	LOH	Pos	2.2
WT055	Female	V	ACC	4.5	I	69	S	No	No	Neg	Neg	NA	LOH	Pos	1.6
WT056	Male	C	ACC	12.8	IV	NA	CT	Yes	Yes	Neg	Neg	NA	LOH	NA	0.5
WT057	Female	V	ACC	1.9	II	190	S	No	No	Neg	Neg	NA	LOH	Pos	4.3
WT058	Female	V	ACC	2.6	III	56	S + CT	No	No	Neg	Neg	NA	LOH	Neg	4.0
WT059	Female	None	ACC	0.8	I	12	S	No	No	Neg	Pos	NA	LOH	Neg	1.6
WT060	Female	V	ACC	11.4	III	156	S + CT	No	No	Neg	Neg	NA	LOH	Neg	1.9

Abbreviations: A, aldosterone-producing tumor; ACA, adrenocortical adenoma; ACC, adrenocortical carcinoma; ACT, adrenocortical tumor; C, Cushing syndrome; CT, chemotherapy; Hypom, hypomethylation; IC2, imprinting control region 2 (KvDMR1 locus); LOH, loss of heterozygosity; N, no copy number change or methylation status alteration; NA, not available; ND, not determined; Neg, negative; Pos, positive; S, surgery; Und, tumor of undetermined histology; UPD, uniparental disomy; V, virilization.

*Acquired ATRX mutation.

†Nuclear positivity for β-catenin.

‡Loss to follow-up.

Table A2. Cox Regression Modeling Showing Association of PFS With Disease Stage, Tumor Weight, Age, and Ki-67 LI Status

Risk Predictor	Hazard Ratio	95% CI	*P*
Stage IV disease	3.905	0.772 to 19.748	.0995
Tumor weight (100-g units)	1.001	1.000 to 1.002	.1929
Age (years)	1.243	1.065 to 1.4511	**.0057**
Ki-67 LI ≥ 15%	41.822	3.514 to 497.779	**.0031**

NOTE. Bold font indicates significance.
Abbreviations: LI, labeling index; PFS, progression-free survival.

Table A3. Characteristics of Patients With ACTs Harboring Mutations or No Mutations in *TP53*

Characteristic	No. (%) Wild-Type *TP53* (n = 60)	No. (%) Mutant *TP53** (n = 54)	*P*
Sex			
Female	42 (70)	38 (70)	
Male	18 (30)	16 (30)	
Age at presentation, years			.05
Median	3.3	2.31	
Range	0.25-21.7	0.56-15.81	
Endocrine signs			**< .001**
Virilization alone	23 (38)	32 (59)	
Virilization plus cushing	10 (17)	17 (31)	
Cushing alone	12 (20)	0 (0)	
No clinical signs	12 (20)	3 (6)	
Aldosterone-producing tumor	3 (5)	1 (2)	
Aldosterone plus cushing	0 (0)	1 (2)	
Tumor histology			**.02**
Adenoma	10 (17)	8 (15)	
Carcinoma	43 (72)	44 (82)	
Undefined	7 (12)	2 (4)	
Ki-67 LI ≥ 15%	27 (48) of 56	21 (84) of 25	
Disease stage			**< .001**
I	18 (30)	19 (38) of 50	
II	14 (23)	8 (16) of 50	
III	13 (22)	21 (42) of 50	
IV	15 (25)	2 (4) of 50	
Tumor weight, g			.44
< 200	39 (68) of 57	26 (55) of 47	
≥ 200	18 (32) of 57	21 (45) of 47	
Treatment			.19
Surgery alone	36 (60)	38 (73) of 52	
Surgery followed by chemotherapy	23 (38)	14 (27) of 52	
Chemotherapy alone	1 (2)	0 (0)	
Molecular markers			
Somatic 11p15 LOH	31 (86) of 36	32 (97) of 33	
Somatic *CTNNB1* mutations	22 (42) of 53	0	
Somatic *TP53* mutations	9 (17) of 54	0	

NOTE. Bold font indicates significance.
Abbreviations: ACT, adrenocortical tumor; LI, labeling index; LOH, loss of heterozygosity.
*Patients registered in the International Pediatric Adrenocortical Tumor Registry.

Table A4. Characteristics of Patients With ACTs and Chromosome 11 Abnormalities

Patient	Phenotype	11p Status	
		Germline	Tumor
WT001	BWS	UPD	ND
WT002		UPD mosaic	mLOH
WT003	Hemihypertrophy	IC2 hypomethylation	LOH
WT005		ci–HHV-6	mLOH
WT009		IC2 hypomethylation	LOH
WT023		IC2 hypomethylation	LOH
WT030		IC2 hypomethylation/ci–HHV-6	mLOH
WT031		UPD	ND
WT043		UPD	ND
WT049	BWS	UPD	ND

Abbreviations: ACT, adrenocortical tumor; BWS, Beckwith-Wiedemann syndrome; ci–HHV-6, human herpesvirus 6 chromosomal integration; IC2, imprinting control region 2 (controls maternally expressed genes *CDKN1C* and *KCNQ1*); LOH, loss of heterozygosity; mLOH, loss of maternal chromosome 11p15; ND, not determined; UPD, uniparental disomy.

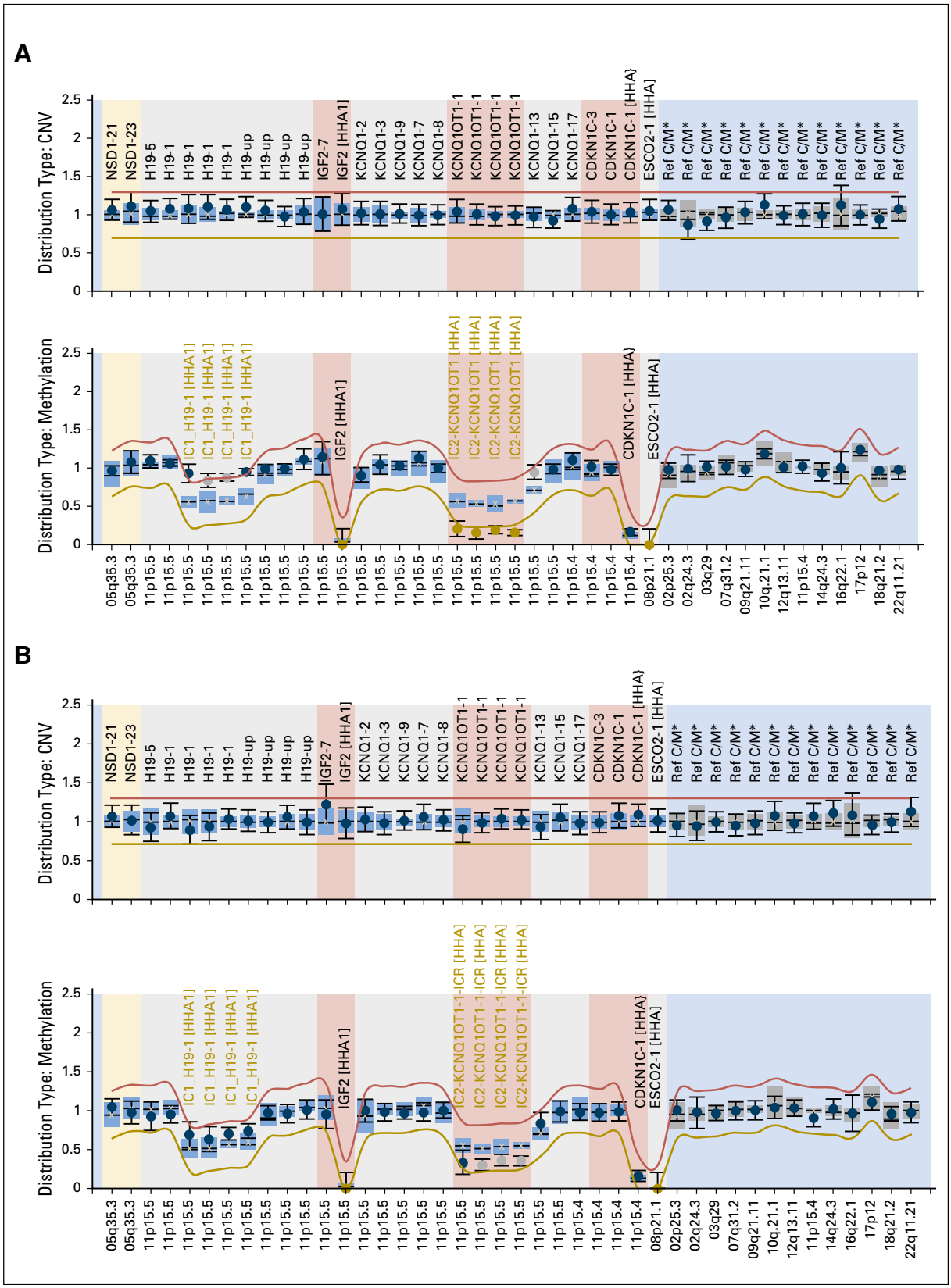

Fig A1. Chromosome 11p15 abnormalities as visualized by methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA) and microsatellite analysis in DNA from pediatric patients with adrenocortical tumors (ACTs) without *TP53* mutations. (A) MS-MLPA analysis of WT031 blood DNA showing (upper panel) normal copy number of chromosome 11p15 and (lower panel) gain of methylation at imprinting control region 1 (IC1) with loss of methylation at imprinting control region 2 (IC2) indicating uniparental disomy. (B) WT002 blood DNA showing partial gain of methylation at IC1 and partial loss of methylation at IC2. (C) MS-MLPA analysis of WT023 blood (continued on next page)

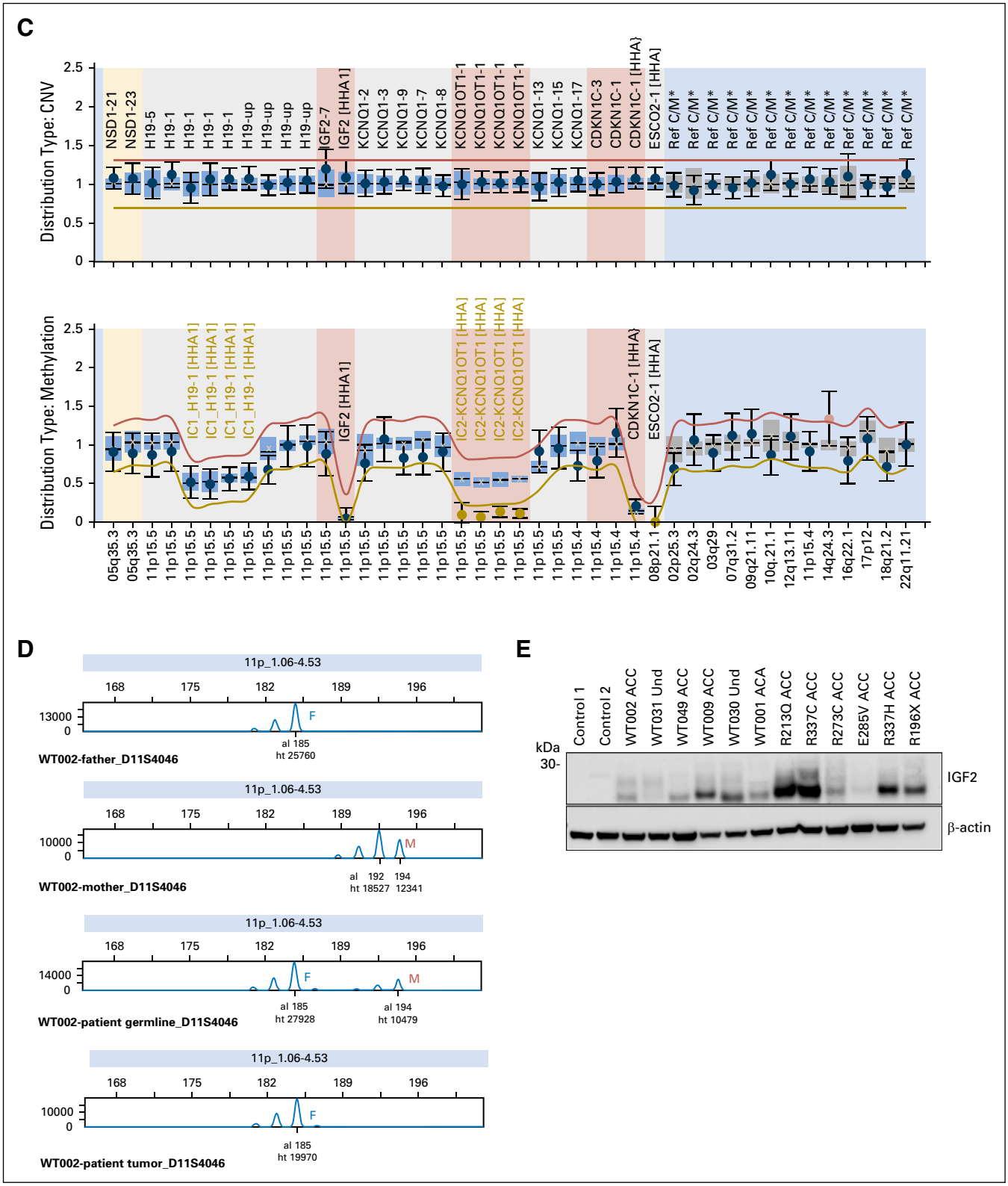

Fig A1. (continued) DNA showing loss of methylation at IC2. (D) Representative microsatellite marker analysis of parents (blood) and patient (blood and tumor) DNA. The father (F) is homozygous (185/185) and the mother (M) heterozygous (192/194) for the D11S4046 marker. Patient blood DNA shows inheritance of alleles 185 from father and 194 from mother; (bottom panel) allele 194 (maternal origin) is selected against in the tumor. (E) Western blot analysis performed with 50 μg of protein with goat polyclonal antihuman antibody directed against insulin-like growth factor 2 (IGF2; 1:500 dilution; Sigma-Aldrich, St Louis, MO) as previously reported.[23] β-actin (1:2,000; Sigma-Aldrich) was used as the loading control. This analysis included patients with 11p15 abnormalities on germline (n = 6) and with germline-mutated *TP53*, as indicated. Levels of *IGF2* were higher in samples than in control (two normal adrenocortical tissues obtained during nephrectomy for Wilms tumor) and were not affected by genotype (wild-type or mutated *TP53*) or histology (carcinoma, adenoma, or undetermined [Und]). ACA, adrenocortical adenoma; ACC, adrenocortical carcinoma; al, allele; CNV, copy number variation; ht, height.

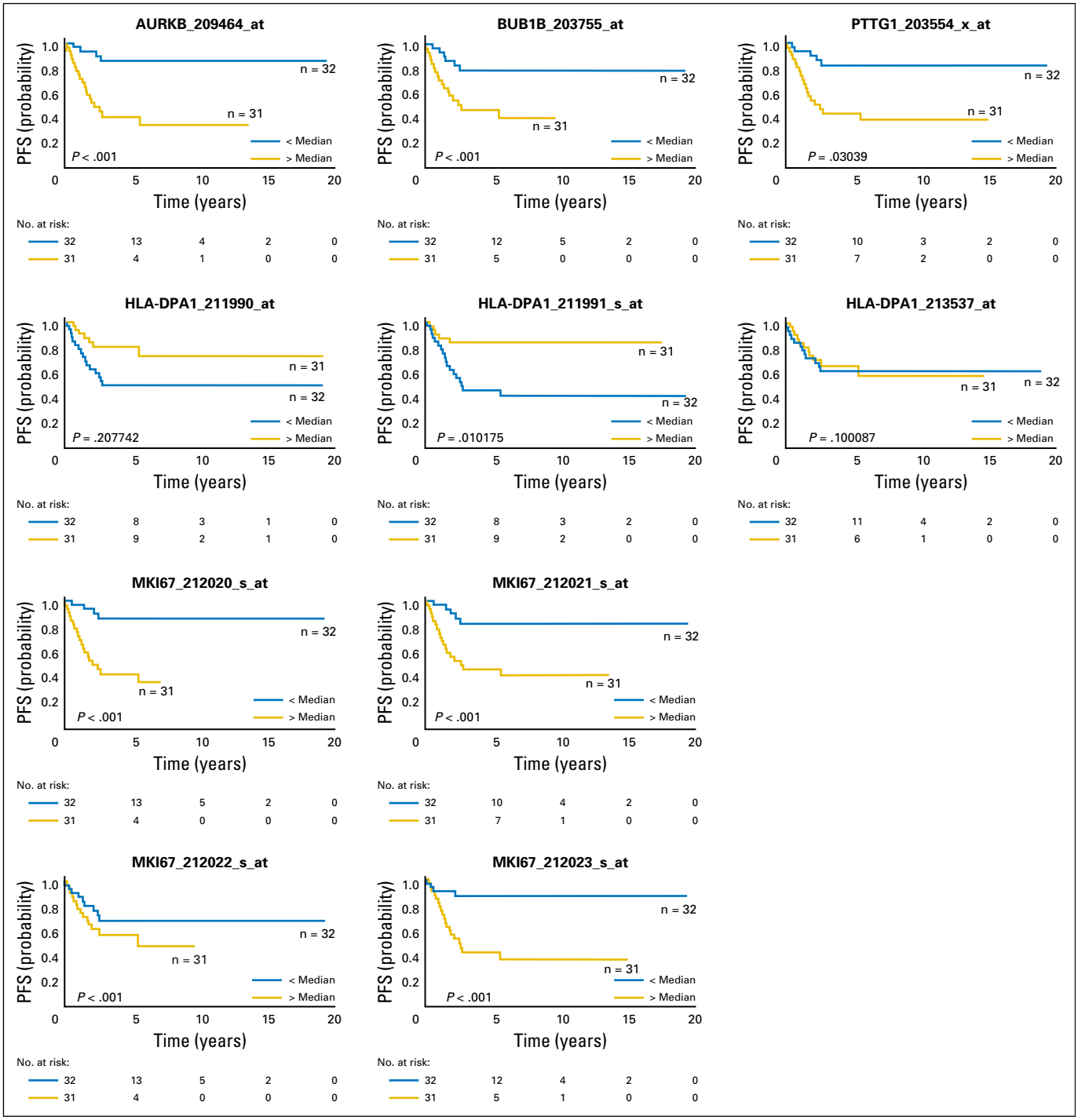

Fig A2. Association of *PTTG1*, *BUB1B*, *AURKB*, *HLA-DPA1*, and *MKI67* mRNA expression with progression-free survival (PFS). mRNA expression was obtained from Gene Expression Omnibus databases GSE76019 and GSE76021. Plots represent mRNA expression according to each probe set for those selected genes. Higher expression of *AURKB*, *BUB1B*, *PTTG1*, and *MKI67* was significantly associated with worse PFS.

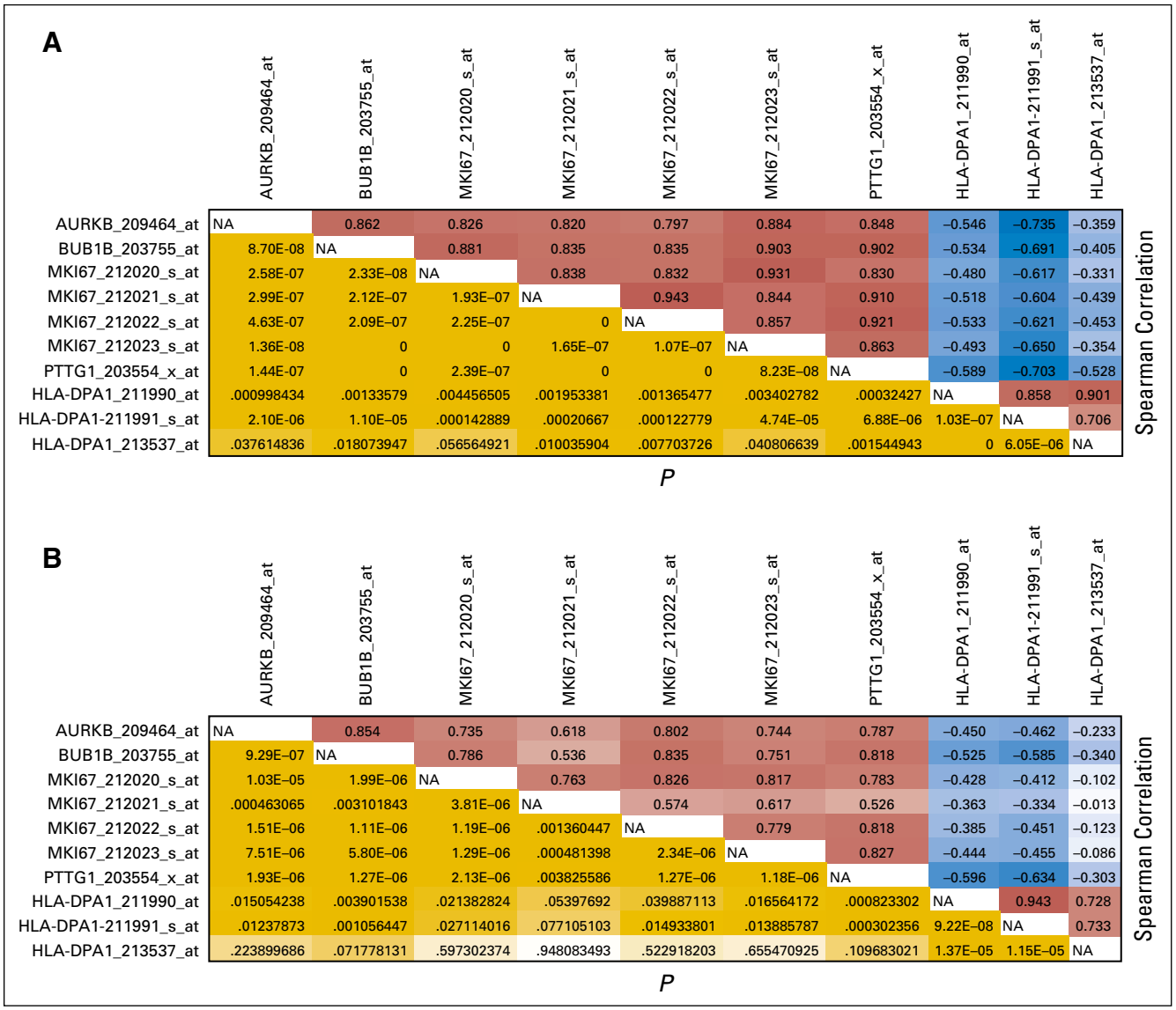

Fig A3. Correlations of mRNA expression of selected cell-cycle genes within the (A) Children's Oncology Group (GSE76019) and International Pediatric Adrenocortical Tumor Registry cohorts (GSE76021). The panels provide the Spearman correlation and *P* value for each pair of cell-cycle gene probe sets in both cohorts. Each entry in the upper triangle gives the Spearman correlation of the expression of the pair of genes indicated by the row and column labels. Each entry in the lower triangle gives the *P* value for the association of the pair of genes indicated by the row and column labels. NA, not applicable.

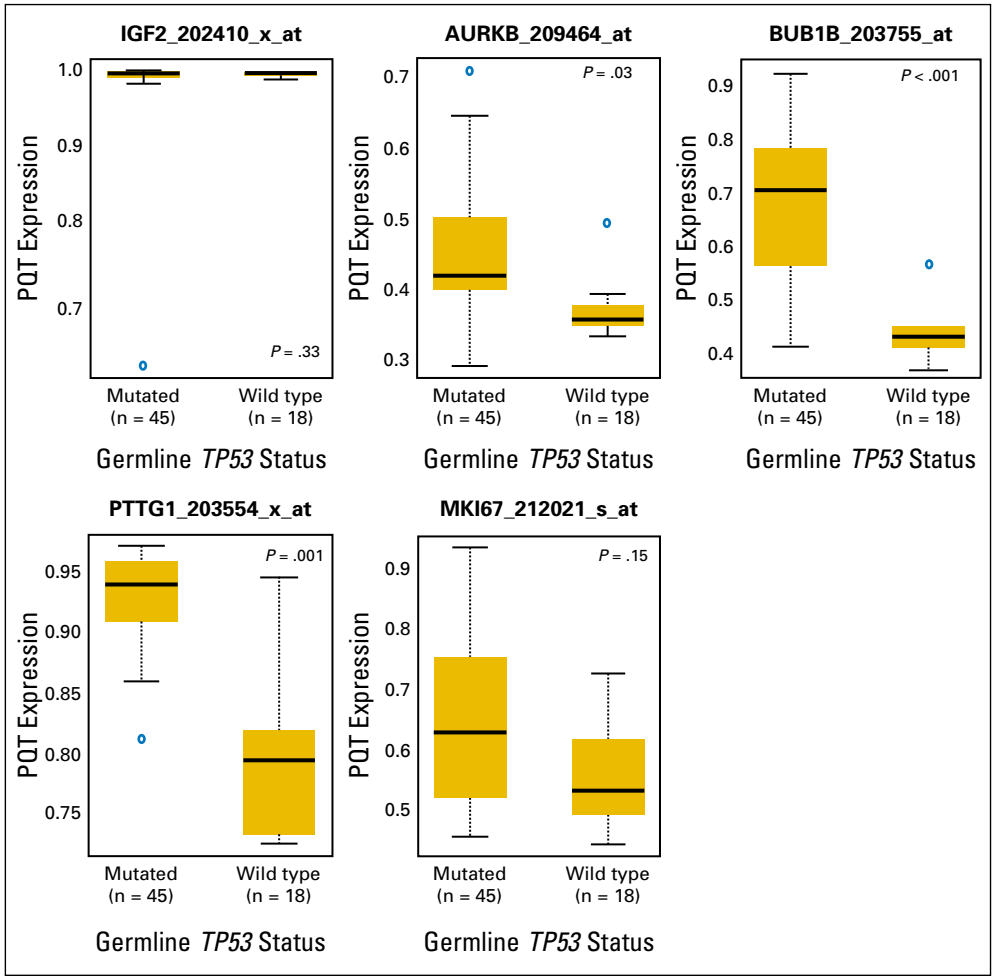

Fig A4. Representative plots of mRNA expression of *MKI67*, *AURKB*, *BUB1B*, and *PTTG1* according to *TP53* status from adrenocortical tumor (ACT) samples in available public databases (Gene Expression Omnibus databases GSE76019 and GSE7602). Expression levels of *PTTG1*, *BUB1B*, and *AURKB* were significantly higher in ACTs with *TP53* mutations than in those without. Expression of *IGF2* and *MKI67* was not significantly different,according to *TP53* status. Expression levels are represented on the positive quantile transformation (PQT) scale.