Low Prevalence of $TP53$ Mutations and $MDM2$ Amplifications in Pediatric Rhabdomyosarcoma

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The tumor suppressor gene $TP53$ is the most commonly mutated gene in human cancer. The reported prevalence of mutations in rhabdomyosarcoma (RMS) varies widely, with recent larger studies suggesting that $TP53$ mutations in pediatric RMS may be extremely rare. Overexpression of $MDM2$ also attenuates p53 function. We have performed $TP53$ mutation/$MDM2$ amplification analyses in the largest series analyzed thus far, including DNA isolated from 37 alveolar and 38 embryonal RMS tumor samples obtained from the Cooperative Human Tissue Network (CHTN). Available samples were frozen tumor tissues ($N = 48$) and histopathology slides. $TP53$ mutations in exons 4–9 were analyzed by direct sequencing in all samples, and $MDM2$ amplification analysis was performed by differential PCR on a subset of 22 samples. We found only one sample (1/75, 1.3%) carrying a $TP53$ mutation at codon 259 (p.D259Y) and no $MDM2$ amplification. Two SNPs in the $TP53$ pathway, associated with accelerated tumor onset in germline $TP53$ mutation carriers, ($TP53$ SNP72 (rs no. 1042522) and $MDM2$ SNP309 (rs no. 2279744)), were not found to confer earlier tumor onset. In conclusion, we confirm the extremely low prevalence of $TP53$ mutations/$MDM2$ amplifications in pediatric RMS (1.33% and 0%, respectively). The possible inactivation of p53 function by other mechanisms thus remains to be elucidated.

1. Introduction

Rhabdomyosarcoma (RMS) is the most common type of soft tissue sarcoma diagnosed in children under the age of 15 years contributing to approximately 4% of all childhood malignancies [1]. Two major subtypes, embryonal (ERMS) and alveolar rhabdomyosarcoma (ARMS), together comprise 80% of all rhabdomyosarcomas [2]. The predominant subtype is ERMS which is characterized by earlier age of onset and better survival compared to ARMS (70% versus 50%, resp.) [2, 3]. While ERMS is characterized by frequent loss of imprinting on chromosome 11p15, a region containing a number of imprinted genes, including $IGF2$, 80% of ARMS present with translocations, most frequently involving $PAX$ 3 or 7 and $FOXO$ gene rearrangements [4, 5]. Young age of onset, a number of identified predisposing syndromes, and paucity of environmental and lifestyle risk factors all contribute to the widely accepted view that genetic aberrations may play an important role in RMS development [3, 5]. However, the etiology of RMS remains largely unknown primarily due to its rarity and diagnostic diversity [2].

$TP53$ is the most commonly mutated gene in human cancer; however, the prevalence of $TP53$ mutations varies greatly by cancer type [6]. This tumor suppressor gene is activated in response to DNA damage and mediates cell cycle
arrest or induction of apoptosis. MDM2 is a key negative regulator of p53 expression; binding of MDM2 to p53 targets p53 for proteasomal degradation. Therefore, overexpression of MDM2 attenuates p53 function.

Missense mutations are the most common mechanisms of TP53 inactivation [7]. Frequently mutations are clustered in exons 5 through 8 [7], the region coding for the DNA binding domain, where “hotspot mutations” have been described. In addition, a number of variants in the TP53 gene have been described, and some of these, including SNP72 (rs no. 1042522, C/G), may modify the risk of cancer development [8]. SNP72 is a coding SNP producing proteins with slightly different properties. In particular, compared to the 72Arg (C allele) variant, the 72Pro (G allele) has a lower affinity for MDM2 [9]. This variant has been shown to affect the age of tumor onset in patients who carry a germline mutation in TP53 [10]. Similarly, accelerated tumor formation in germline TP53 carriers was observed in carriers of the minor allele (G) for the MDM2 SNP309 [11, 12]. The TP53 SNP72 C allele has been shown to interact with the MDM2 SNP309 G allele to amplify the risk of tumor onset at an earlier age in carriers [10].

The risk of RMS development increased in TP53 mutation carriers, but the role of the TP53 polymorphisms described above on the age of cancer onset has not been explored in sporadic RMS.

TP53 mutations have been studied in RMS, but the majority of these studies had small sample sizes and the prevalence of reported mutations varied greatly (Table 1) [13–24]. Larger studies have also reported inconsistent data. For example, Mulligan et al. [18] and Taylor et al. [22] detected very low frequencies of TP53 mutations, 2/31 and 1/20, respectively, while Takahashi et al. [21] reported a much higher mutation frequency (9/45). In addition, Taylor et al. [22] examined amplifications of the MDM2 gene, a frequent mechanism for MDM2 overexpression in other tumor types, and detected a very low frequency of amplifications (2/20).

We have analyzed TP53 mutations in the largest series of RMS tumors thus far, enabling the analyses by RMS subtypes, as well as MDM2 amplification status. In addition, we have examined whether TP53 and MDM2 polymorphisms may affect the age of RMS onset and have also explored the associations between these polymorphisms and specific tumor subtype and primary tumor site.

2. Material and Methods

2.1. Tumor Samples. Deidentified frozen RMS tumor tissues (N = 40) were obtained from the Cooperative Human Tissue Network (CHTN). Histopathology slides (N = 40) were obtained separately from CHTN and kindly provided for this study by Dr Brenda Weigel (University of Minnesota). Five of the histology slides corresponded to five of the frozen tumor tissues, and therefore the final sample collection included 75 different RMS patient tumors. CHTN houses the tissues of Children’s Oncology Group’s (COG) pediatric solid tumor bank, obtained after routine surgical resections performed at COG-affiliated institutions across the United States and Canada. Tumors were preserved either by snap-freezing in liquid nitrogen and storing at −80°C or by fixation in formalin, followed by paraffin embedding for histopathology slides. Central review of histologic slides and clinicopathologic data was performed by an anatomic pathologist at CHTN for each sample, and histologic subtypes (embryonal or ERMS; alveolar or ARMS) were determined. For a handful of samples, information on percentage of area of tissue involved by tumor and percentage of necrotic tumor cells was available. Review of all histopathology slides was performed by Carlos Manivel, M.D., at the University of Minnesota to estimate the percentage of tumor and necrosis in the samples.

2.2. Sequencing of the TP53 Gene. DNA was isolated from frozen tissues by using DNasy blood and tissue mini kit, while DNA from histopathology slides was isolated using QIAamp DNA Micro kit, according to the manufacturer’s protocol (Qiagen, Valencia, CA). DNA amplification and TP53 sequencing were performed at the International Agency for Research on Cancer (IARC) common sequencing service using standard procedures. Primer sequences, product lengths, and PCR conditions are available at http://www-p53.iarc.fr/Download/TP53_DirectSequencing_IARC.pdf. PCR products were purified using the enzyme ExoSap IT (USB), and 7 μL of each purified product was sequenced by using BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems) according to manufacturer’s protocol. Sequencing primers were the same as the ones used for amplification. Sequencing reaction products were purified by using 96-well Multiscreen filtration plates (G50-Pharmacia-Millipore) and analyzed by a 16-capillary automated sequencer (ABI PRISM 3100 Genetic Analyzer, Applied Biosystems).

2.3. Analysis of MDM2 Amplifications. MDM2 amplification was analyzed by using differential PCR. The analysis was carried out as previously described, with some modifications [25]. Briefly, each DNA sample isolated from 22 frozen tissue samples was added to a PCR mix with primers for MDM2 and primers for dopamine receptor 2 (DRD2) reference sequence. The sequences of the primers were 5′-GAGGGCTTTGATGTCTTCTGA-3′ (forward) and 5′-GCTACTGAAGTTGATGG C-3′ (reverse) for MDM2, and 5′-CCACTGAACTGTCTGATG-3′ (forward) and 5′-GTGTGG CATAGTAGAGTGGT G-3′ (reverse) for DRD2. PCR products were analyzed by electrophoresis on 3.0% agarose gel and stained with ethidium bromide. The intensities of the DNA products were quantified by densitometry using ImageQuant Version 5.0 software (Molecular Dynamics, Inc., Sunnyvale, CA). The MDM2/DRD2 ratio from normal blood DNA was 0.9, with a standard variation of 0.23. A value of more than 2.5 (2x mean + 3x SD) for the MDM2/DRD2 ratio was regarded as positive for MDM2 amplification [25].

2.4. Analysis of MDM2 SNP309. DNA samples were genotyped for the single nucleotide polymorphism (SNP) MDM2 309 using the 5′ nulease Taqman allelic discrimination assay including Taqman Universal PCR Master Mix (Applied Biosystems Inc., ABI, Foster City, CA), as previously described [11]. For each assay, there were four no template controls,
2.5. Statistical Methods. Patient tumor characteristics and SNP classifications were compared using Chi-squared and Fisher’s exact tests as appropriate for categorical data and t-tests and analysis of variance (ANOVA) for continuous variables. Means ± standard deviations (SD) are reported unless otherwise noted. A P-value of <0.05 was considered statistically significant. All analyses were performed using SAS version 9.2.

3. Results

3.1. Characterization of Patient Tumors. Clinicopathologic data included histologic subtype, primary site, age at diagnosis, gender, and race/ethnicity (Table 2). The majority of patients were non-Hispanic Caucasians (74.4%), while 6.7% of patients were Hispanic, and 9.3% were African Americans. There were 45 male and 30 female pediatric RMS patients ranging in age from 3 months to 18 years. There was no difference in mean age by histologic subtype: age for ERMS was 9.0 ± 3.0 years and for ARMS was 6.1 ± 2.6 years. A large diversity of primary sites showed that the tumors could arise almost anywhere in the body. When the sites were grouped, extremities represented the single most common site (N = 19; 25.3%), followed by genitourinary (N = 18; 24.0%), head and neck (N = 11; 14.7%), and abdomen (N = 9; 13.3%) (Table 2). Tumors occurring in extremities had predominantly ARMS histology (15/19), while genitourinary tumors had predominantly ERMS histology (15/18). Tumors in trunk and visceral locations all had ARMS histology.

The age at diagnosis differed significantly with primary site (P = 0.035). The youngest age at diagnosis was observed for pelvic tumors (mean age ± standard deviation was 2.8 ± 1.0 years, resp.) and the oldest for genitourinary tumors (10.2 ± 5.0 years). Patients with abdominal tumors were significantly younger at diagnosis (P = 0.05). We also observed a significant difference (P = 0.012) between tumor primary sites by gender, with genitourinary and abdominal tumors predominantly observed in males (15/18 and 8/10, resp.) and head and neck tumors in females (9/11).

Seventy five RMS tumor samples were analyzed for TP53 mutations in exons 4–9 by direct sequencing. We identified only a single TP53 mutation present in exon 7, c.775G/T (p.D259Y). This missense mutation leads to loss of p53 function. The patient harboring this mutation was a one-year-old Caucasian boy with ERMS tumor on the right thigh, tumor stage was not obtained. By analyzing sequencing data, we have also observed a number of variants in the TP53 gene. In addition to SNP72, the most prevalent SNP was rs1004–1017 (p.D259Y). This missense mutation leads to loss of p53 function. The patient harboring this mutation was a one-year-old Caucasian boy with ERMS tumor on the right thigh, tumor stage was not obtained. By analyzing sequencing data, we have also observed a number of variants in the TP53 gene. In addition to SNP72, the most prevalent SNP was rs1004–1017 (p.D259Y). This missense mutation leads to loss of p53 function. The patient harboring this mutation was a one-year-old Caucasian boy with ERMS tumor on the right thigh, tumor stage was not obtained. By analyzing sequencing data, we have also observed a number of variants in the TP53 gene.

| Study size | Authors | Exons sequenced | Number of mutations | Location of mutations |
|------------|---------|-----------------|---------------------|----------------------|
| ≤10        | Stratton et al., [20] | 5–8 | 0/4 | — |
|            | Felix et al., [15] | 4–8 | 1/6 | Exon 6: R213P |
|            | Toguchida et al., [23] | 2–11 | 0/4 | — |
|            | Latres et al., [17] | 2–9 | 0/2 | — |
|            | Castresana et al., [13] | 5–8 | 1/1 | Exon 6: V218Y Y220C Exon 11: D393N |
|            | Würl et al., [24] | 4–9 | 2/6 | Exon 7: G245S |
|            | Kusafuka et al., [16] | 5–8 | 1/10 | Exon 8: R273H |
|            | Nawa et al., [19] | 5–8 | 0/2 | — |
|            | Das et al., [14] | 2–11 | 1/4 | — |
| >10        | Mulligan et al., [18] | 5–8 | 2/31 | Exon 5: G137V Exon 8: R282W |
|            | Taylor et al., [22] | 5–9 | 1/20 | Exon 5: del nt 1004–1017 Exon 6: E204G, R209T, P223R |
|            | Takahashi et al., [21] | 5–9 | 9/45 | Exon 7: M243T, G245C, N247D, R249G Exon 8: C291G, P295H |
| Total TP53 mutations | 18/135 (13.3%) |

Table 1: Frequency of TP53 point mutations reported in rhabdomyosarcoma.

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We have also analyzed the polymorphisms in MDM2 SNP309 and TP53 SNP72 in DNA isolated from frozen-tumor samples (N = 40) (Table 3). For MDM2 SNP309 we observed the following genotype frequencies: 40% for T/T, 40% for T/G, and 20% for G/G. These frequencies are similar to those previously reported in healthy Caucasians [11, 26, 27]. The mean age of diagnosis was 6.4 years for T/T, 6.7 years for T/G, and 7.6 years for G/G MDM2 SNP
alleles and are not statistically different from each other ($P = 0.85$). For the TP53 SNP72, the frequencies of C/C, C/G, and G/G genotypes in rhabdomyosarcoma were 60%, 30%, and 10%, respectively, which were similar to those in healthy Caucasians [28, 29]. Although the mean age of tumor onset in C/C genotype carriers was slightly lower compared to those with C/G or G/G genotypes (6.3 ± 5.2 versus 7.5 ± 4.8, and 7.0 ± 4.8, resp.), these differences were not significant ($P = 0.78$). We have also analyzed the effect of either polymorphism (MDM2 SNP309 or TP53 SNP72) on the tumor subtype and observed no association. Likewise, there was no association between any SNP and tumor primary site.

Because MDM2 SNP309G and TP53 SNP72Arg have been reported to act synergistically and were together associated with the lowest age of cancer onset in carriers [10], we examined whether these variants may act together in sporadic RMS to confer increased risk of cancer onset at a younger age (Table 4). Only a single patient possessed the protective genotype, reported to be associated with later cancer onset in germline TP53 mutation carriers [10] (0 at risk loci shown in Table 4), and although patients with less favorable genotypes appeared to be slightly younger at cancer diagnosis, these differences were not statistically significant ($P = 0.78$).

### 4. Discussion

A wide range of prevalences of TP53 mutations in RMS were reported in the literature, with larger studies tending to report lower mutation frequencies. To address these inconsistencies, we have analyzed TP53 mutations in exons 4–9 in 75 pediatric RMS tumor samples, as the majority of TP53 mutations occur in exons 5–8. We detected only a single mutation in exon 7 (c.775G/T, p.D259Y), associated with loss of p53 activity [30]. This occurred in a 1-year-old patient with ERMS. ERMS are characterized by an earlier age of onset compared to ARMS, with approximately half of all cases diagnosed before the age of 5 years. Interestingly, the tumor of this patient was located in the lower extremity, a site that is more frequently associated with ARMS tumors.

All tumor samples in this study came from the CHTN, which includes a central pathology review. However, the majority of samples did not have accompanying data on the percentage of tumor present in each sample or the prevalence of necrosis. We therefore performed hematoxylin staining of the available histopathology slides and reviewed all histopathology slides to complete these data. This helped us address the possibility that the observed low prevalence of mutations in these tumors may be due to a large content of normal tissue compared to tumor tissue in our samples, which might undermine the detection of mutations that may be present in tumor. However the pathology review determined that, except for two samples, that had approximately 60% of the sample composed of tumor tissue, at least 80% of all other samples was tumor tissue. All reviewed samples had over 90% viable cells present. Therefore, it is unlikely that the composition and integrity of our samples may have affected the observed low prevalence of TP53 mutations.

Differences in the methods used to identify mutations in earlier compared to more recent studies may partially account for the observed differences in mutation frequencies. All earlier studies used single strand conformation polymorphism (SSCP), a method shown to more frequently detect TP53 mutation detection, compared to sequencing, which is current gold standard [31, 32].

Some clinicopathologic characteristics of our samples were in line with well-established differences between the two major RMS subtypes, including differences in primary sites, and equal male to female ratio among ARMS cases [2]. In contrast, earlier age of onset of ERMS compared to ARMS was not reflected in our samples (Table 2). Despite slightly older age of ERMS cases than anticipated, the proportion of very young children (up to 5 years of age) was substantial (34/75), indicating that the age may not explain the observed low mutation prevalence. Namely, among germine TP53 carriers, RMS occurred very early in life and many developed RMS before the age of 5 years [33]. By analogy, if sporadic RMS tumors harboring TP53 mutations were more likely to occur in younger children, a study not including this age category would likely report lower prevalence of TP53 mutations. There was a disproportionately higher number of males than females with ERMS in our study compared to the reported 1.3 male to female ratio. As we observed no gender differences, gender distribution was unlikely to affect our results.

To explore whether increased levels of MDM2 may provide an alternative mechanism to TP53 gene mutation, we have analyzed MDM2 gene amplifications. Overexpression

### Table 2: Patient and tumor characteristics.

| Characteristics     | Histologic subtype | |
|---------------------|--------------------|---|
|                     | ARMS ($N = 37$)    | ERMS ($N = 38$) |
| Sex                 | Male: 18           | 27 |
|                     | Female: 19         | 11 |
| Mean age (STDEV), years | Boys: 5.5 (4.6)   | 7.4 (5.0) |
|                     | Girls: 8.5 (5.3)   | 6.0 (5.1) |
| Race/ethnicity      | Caucasian: 26      | 30 |
|                     | African American: 3 | 4 |
|                     | Hispanic: 2        | 3 |
|                     | Other: 6           | 1 |
| Tumor site          | Head and neck: 7   | 4 |
|                     | Upper extremity: 5 | 1 |
|                     | Lower extremity: 10| 3 |
|                     | Trunk: 3           | 0 |
|                     | Abdomen: 2         | 8 |
|                     | Visceral: 4        | 0 |
|                     | Retroperitoneal: 2 | 4 |
|                     | Pelvic: 1          | 3 |
|                     | Genitourinary: 3   | 15 |
or amplification has been observed in a number of human cancers [34, 35] suggesting that it may act as an alternative mechanism to attenuate wild-type p53 function. Although MDM2 amplifications were frequently observed in soft tissue sarcoma [34, 35], the only study examining this in pediatric RMS has shown very low prevalence of MDM2 amplifications (2 out of 20 samples) [22]. In addition, a study that included both pediatric and adult RMS patients showed a similar prevalence (3 out of 18 patients) [21].

Our study confirms the finding that the prevalence of MDM2 amplifications in pediatric RMS may be low, as we detected no MDM2 amplifications in the 22 samples we analyzed. In contrast to the previous suggestion that MDM2 amplifications may be more likely found in ARMS tumors [21], we observed no amplifications in either tumor subtype.

Among the variants that affect p53 degradation, MDM2 SNP309 was associated with earlier age of cancer onset among germline TP53 mutation carriers [10]. In addition, this SNP was reported to be associated with younger age of onset of sporadic soft tissue sarcoma [11], but this study included insufficient number of RMS samples to determine whether this association would be also observed for RMS. In contrast, our study included a much larger number of pediatric RMS samples, and we found no association between MDM2 SNP309 and younger age of tumor onset. Likewise, TP53 SNP72 was reported to be associated with age of tumor onset in carriers, where it was also shown to interact with MDM2 SNP 309 to further reduce the age of tumor onset. Such interactions have also been described in several sporadic human malignancies; therefore, we examined whether TP53 SNP72 may act as a modifier in RMS. We found no effect of TP53 SNP72 on the age of tumor diagnosis, nor any evidence of interaction between TP53 SNP72 and MDM2 SNP309 on the age of tumor onset.

Although our study was relatively large, it was focused on pediatric RMS. Therefore, the range of ages at tumor onset was narrower compared to other studies that reported modifying effects of the variants affecting p53 degradation in either germline TP53 carriers or other types of sporadic cancer. In addition, molecular characteristics of RMS tumors that occur in children may differ from those that occur in adults. Therefore, it remains possible that any modifying effects of the variants analyzed here would have been observed if adult cases had been included in our study. Further studies involving both pediatric and adult RMS patients would be needed to explore this.

In conclusion, we observed low prevalence of TP53 mutations and no MDM2 amplifications in pediatric RMS. This is different from what has been found before, and due to the sample size, ours is likely a more accurate finding. Variants TP53 SNP72 and MDM2 SNP309 did not accelerate tumor development. TP53 may therefore not play an important role in pediatric RMS development. The possible inactivation of p53 function by other mechanisms thus remains to be elucidated.

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