ORIGINAL ARTICLE

Mutational spectrum of *ATRX* aberrations in neuroblastoma and associated patient and tumor characteristics

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Abstract

Neuroblastoma is the most common extracranial solid tumor in children. The chromatin remodeler *ATRX* is frequently mutated in high-risk patients with a poor prognosis. Although many studies have reported *ATRX* aberrations and the associated clinical characteristics in neuroblastoma, a comprehensive overview is currently lacking. In this study, we extensively characterize the mutational spectrum of *ATRX* aberrations in neuroblastoma tumors reported in previous studies and present an overview of patient and tumor characteristics. We collected the data of a total of 127 neuroblastoma patients and three cell lines with *ATRX* aberrations originating from 20 papers. We subdivide the *ATRX* aberrations into nonsense, missense, and multiexon deletions (MEDs) and show that 68% of them are MEDs. Of these MEDs, 75% are predicted to be in-frame. Furthermore, we identify a missense mutational hotspot region in the helicase domain. We also confirm that all three *ATRX* mutation types are more often identified in patients diagnosed at an older age, but still approximately 40% of the patients are aged 5 years or younger at diagnosis. Surprisingly, we found that 11q deletions are enriched in neuroblastomas with *ATRX* deletions compared to a reference cohort, but not in neuroblastomas with *ATRX* point mutations. Taken together, our data emphasizes a distinct *ATRX* mutation spectrum in neuroblastoma, which should be considered when studying molecular phenotypes and therapeutic strategies.

KEYWORDS
11q deletion, ALT, ATRX, neuroblastoma, pediatric oncology

1 | INTRODUCTION

Neuroblastoma is the most common extracranial solid tumor in children and has an incidence of 10.2 cases per million under the age of 15 years.[1,2] It arises from progenitor cells during fetal adrenal neuroblast development.[3] Clinical, genetic, and pathologic characteristics are used for risk stratification and treatment allocation.[4] Although the 5-year overall survival rate for patients has improved over recent years to 81%, for patients with high-risk disease survival remains 50% despite multimodal treatment regimens.[1]

Abbreviations: ALT, alternative lengthening of telomeres; B-ALL, B-cell acute lymphoblastic leukemia; IFD, in-frame deletion; IFF, in-frame fusion; MED, multiexon deletion; MNA, *MYC-N* amplification; SCA, segmental chromosomal aberration; XCI, X chromosomal inactivation.

High-risk tumors can be subclassified in four categories based on genomic aberrations: *MYCN* amplifications (37%), *TERT* rearrangements (23%), *ATRX* deletions (11%), and having none of the previously mentioned aberrations.[5,6] All these genetic aberrations are independently associated with a poor clinical outcome for patients. Both MNA and *TERT* rearrangements induce elevated expression of *TERT* and thereby facilitate telomere maintenance, one of the hallmarks of cancer.[5] In contrast, *ATRX* aberrations can induce ALT, which is a homologous recombination-based telomere maintenance mechanism that occurs in 5–15% of cancer.[7] The MNA/*TERT* rearrangements and *ATRX* aberrations are mutually exclusive, whereas MNA and *TERT* rearrangements sporadically co-occur.[5]

ATRX, located on the X-chromosome, is a gene that encodes for an ATP-dependent helicase of the SWI/SNF family of chromatin remodelers. The coding sequence, excluding the introns, comprises 7479 base pairs and consists of 35 exons. ATRX forms a chromatin remodeling complex with DAXX, which performs the replication-independent deposition of the histone variant H3.3 at pericentromeric and telomeric regions.[8,9] In addition, ATRX is both directly and indirectly involved in a plethora of nuclear processes, such as removing G-quadruplexes,[10] inhibiting macroH2A1 deposition,[11] removing R-loops,[12] modifying histones,[13] methylating DNA,[14] and resolving replication fork stalling.[15]

This wide spectrum of *ATRX* functions might be reflected by the large shift in the expression of hundreds of genes that has been observed in cell lines derived from neuroblastoma patients with large MEDs resulting in IFDs of the *ATRX* gene.[16] These IFDs do not lead to an absence of protein, but rather result in a shorter protein product. In neuroblastoma, both *ATRX* MEDs and point mutations have been reported, and these aberrations are strongly associated with ALT development[5,6,16-34]. *ATRX* aberrations are also present in various other pediatric[25,31,35-56] and adult cancers.[57]

Although several studies investigating genomic alterations in neuroblastoma have reported *ATRX* aberrations, the number of patients was often limited and a comprehensive analysis of the spectrum of *ATRX* mutations and patient characteristics is currently lacking. Therefore, in this meta-analysis we extensively characterized the mutational spectrum of *ATRX* aberrations in neuroblastoma tumors described in previously published works and compared these with other pediatric cancers. Furthermore, we present an overview of the patient characteristics and co-occurring aberrations in this neuroblastoma subgroup.

2 | MATERIALS AND METHODS

2.1 | Data acquisition and analysis

To obtain all reported pediatric cancers with known genetic *ATRX* aberrations, we carried out a thorough search in several databases. The last search was undertaken on April 30, 2020 and all identified papers were manually assessed for patients with known genetic *ATRX* aberrations. Twenty-one tumors with *ATRX* deletions

described by Brunner et al.[19] and Abbasi et al.[17] were reanalyzed for this study for breakpoints and deleted exons. From all identified papers with neuroblastoma patients with *ATRX* aberrations, we collected the specific genetic mutation and a selection of the tumor/patient data (Table S1). The *ATRX* aberrations were reported in our paper based on the human reference genome Hg19 and transcript variant 1 (NM_000489.6). As multiple published papers can use the same dataset, we carefully assessed the origin of each patient and in that manner removed duplicates. To create the overviews of the *ATRX* point mutations and MEDs we used the genomic positions for plotting. For the analysis of the predicted effect of the missense mutation we performed PROVEAN protein batch analysis[58] and for every analysis on the patient and tumor data all available input data were used (Table S1) and missing data were removed per analysis. All the data were analyzed and plotted using R 4.0.2[59] and ggplot2_3.3.5.[60] For further details see Document S1.

3 | RESULTS

3.1 | *ATRX* aberrations in neuroblastoma and other pediatric cancers

After collecting all the data on *ATRX* aberrations from neuroblastoma reports, we identified 127 unique patients and three cell lines from a total of 20 papers and belonging to 16 cohorts. Apart from two tumors, that were stage 2 and 3, all tumors were classified as stage 4. For comparison we also included the *ATRX* aberrations identified in other pediatric cancers of 122 tumors and one cell line (Table S1). These data originated from 23 studies and comprised nine different tumor types, mainly osteosarcoma, high-grade glioma, or B-ALL (Figure 1A). Tumors with *ATRX* aberrations can be divided in three subgroups based on their mutation type: nonsense/frameshift mutations, missense mutations, or MEDs. We defined MEDs as deletions involving at least two complete exons; all reported smaller deletions induce frameshifts and were therefore included within the nonsense/frameshift category. We observed that 68% of the neuroblastoma samples contained MEDs, whereas the MEDs only comprised 8% in other pediatric cancers, with the majority belonging to osteosarcomas (Figure 1B and Table S1). This indicated that the MEDs are almost exclusively present in neuroblastoma. Furthermore, these data showed that point mutations were less frequent in neuroblastoma, only constituting 32% of the cases, while making up to 92% of the cases in other pediatric cancers (Figure 1B). Overall, this indicates that *ATRX* aberrations in neuroblastoma have a different distribution of mutation types, with a high frequency of MEDs.

3.2 | Identification of a mutational hotspot region in the helicase domain for *ATRX* missense aberrations in neuroblastoma

To further analyze the *ATRX* mutational spectrum in neuroblastoma and other pediatric cancers, we determined the frequency and
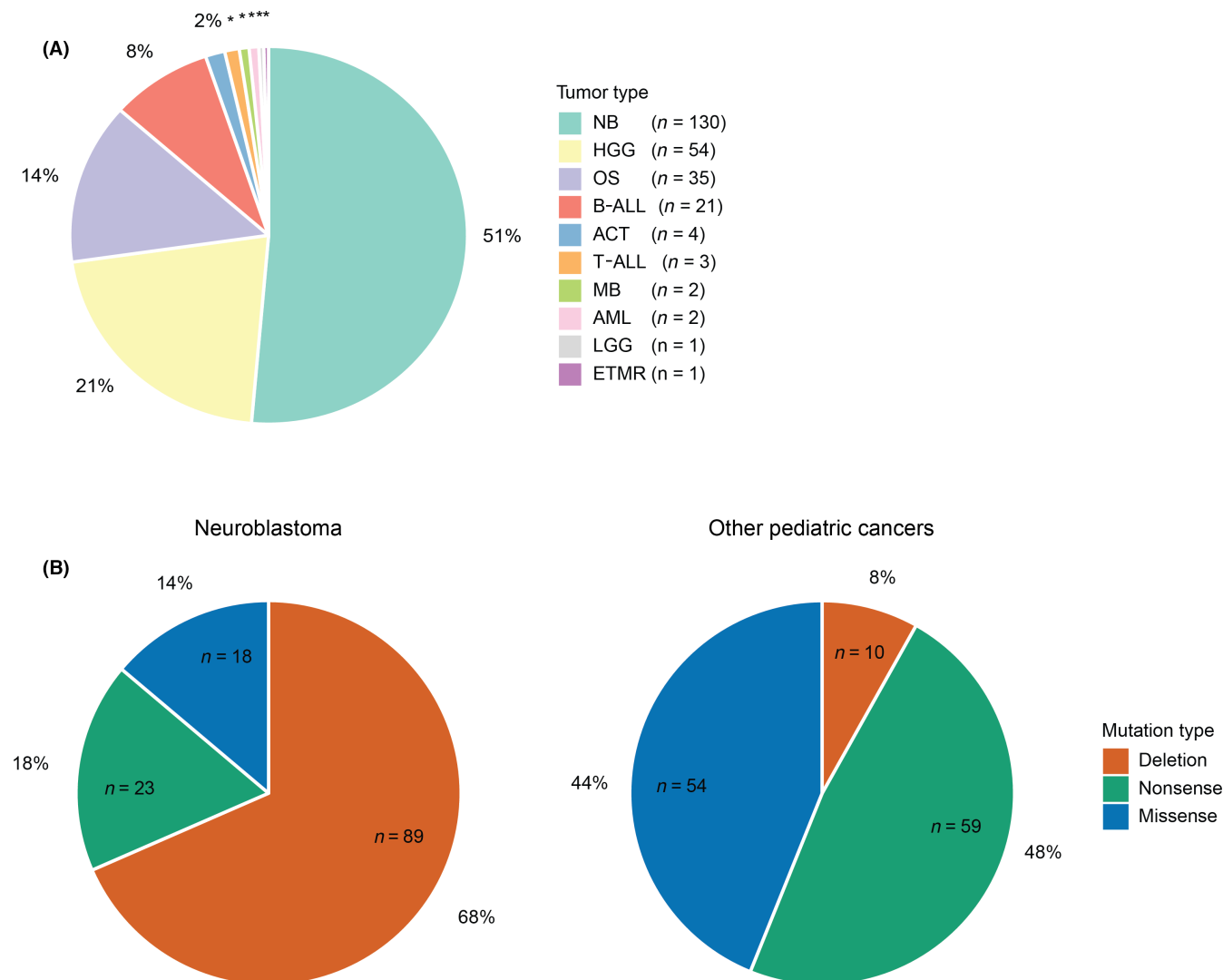
FIGURE 1 Patient and mutation type distribution in our compiled dataset. (A) Distribution in percentages of all patients within our dataset across the different pediatric cancer types. ACT, adrenocortical tumor; AML, acute myeloid leukemia; B-ALL, B-cell acute lymphoblastic leukemia; ETMR, embryonic tumor with multilayered rosettes; HGG, high-grade glioma; LGG, low-grade glioma; MB, medulloblastoma; NB, neuroblastoma; OS, osteosarcoma; T-ALL, T-cell acute lymphoblastic leukemia. *≤1%. (B) Distribution in percentages of the three *ATRX* mutation types in neuroblastoma (left) and all other pediatric cancers (right)

distribution of nonsense/frameshift mutations as well as missense mutations across the gene.

For neuroblastoma, 23 nonsense/frameshift *ATRX* mutations were reported, whereas 59 cases were described for all other pediatric cancers (Table S1). These aberrations were randomly distributed across the *ATRX* gene, which was also the case for the other pediatric cancers (Figure 2A). Recurrent nonsense/frameshift *ATRX* mutations were very rare and none of the recurrent mutations occurred in more than two patients. The only exception was R2386* (Table S1), which was present in three osteosarcoma patients, including two siblings with a germline mutation.[50]

In addition, *ATRX* missense mutations were identified in 17 neuroblastoma tumors and one cell line and 54 other pediatric tumors (Table S1). When these mutations were mapped against the *ATRX* gene, we observed a mutational hotspot region for neuroblastoma

in the helicase domain (56% of missense mutations), especially the C-terminal helicase domain where nearly 45% of all reported missense mutations were located (Figure 2B). Missense mutations were significantly enriched in the helicase domain for neuroblastoma (binomial test, $p = 8.633\text{e-}06$). Furthermore, 38% and 36% of all *ATRX* missense mutations for high-graded glioma and osteosarcoma, respectively, were also restricted to the helicase domains. In summary, this strongly supports the presence of a mutational hotspot region within the *ATRX* helicase domain across pediatric cancers.

To date no complete 3D structure of the ATRX protein exists, therefore we were unable to assess structural consequences of the missense mutations and were limited to mutation prediction software to indicate potential biological effects. Of the 18 missense mutations identified in neuroblastoma tumors, 13 were predicted to have a deleterious effect by both the SIFT and PROVEAN analyses
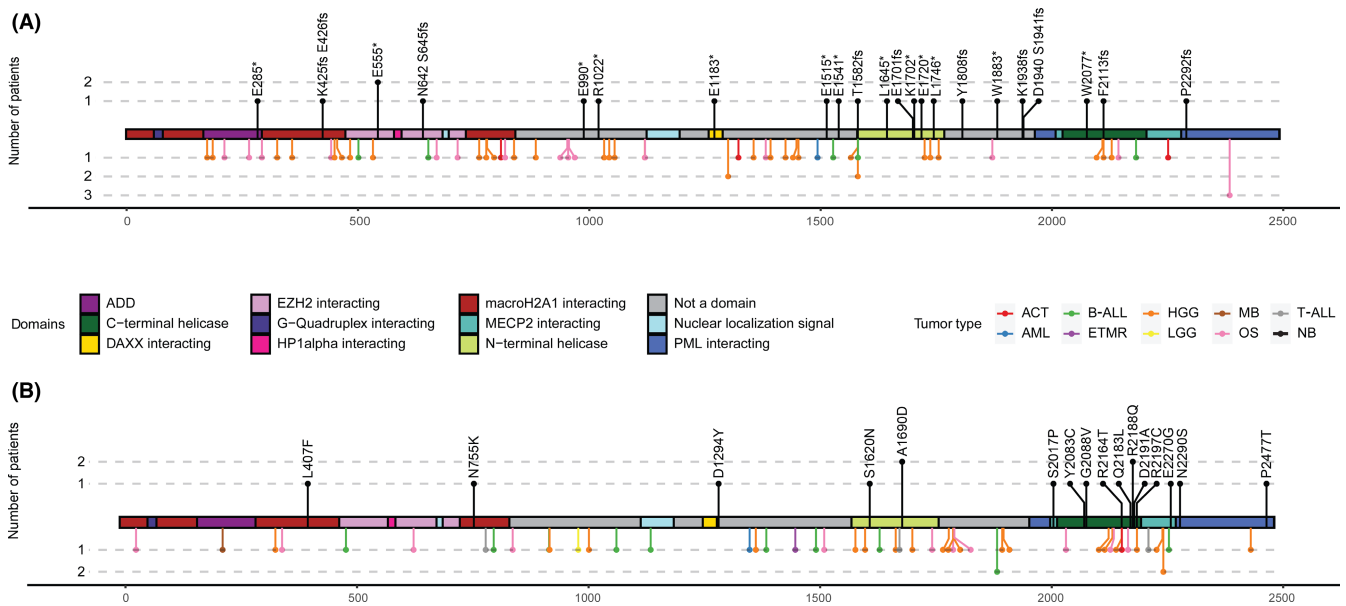
(A)

(B)

FIGURE 2 Pediatric point mutation landscape within the *ATRX* gene. (A) Distribution of nonsense and frameshift mutations of all pediatric cancers within our dataset across the *ATRX* gene. (B) Distribution of missense mutations of all pediatric cancers within our dataset across the *ATRX* gene. In both panels, neuroblastoma mutations are displayed above the bar and below the bar mutations that were identified in other pediatric cancers are displayed. All mutations were plotted based on DNA positions. ACT, adrenocortical tumor; AML, acute myeloid leukemia; B-ALL, B-cell acute lymphoblastic leukemia; ETMR, embryonic tumor with multilayered rosettes; HGG, high-grade glioma; LGG, low-grade glioma; MB, medulloblastoma; NB, neuroblastoma; OS, osteosarcoma; T-ALL, T-cell acute lymphoblastic leukemia

(Table S2) and two were reported as neutral by both algorithms. Overall, we can conclude that *ATRX* missense mutations are predominantly present in the helicase domain and that they are predicted to disturb protein function, whereas nonsense mutations are randomly distributed across the gene and could result in absence of ATRX protein production.

3.3 | Majority of recurrent *ATRX* MEDs in neuroblastoma are predicted in-frame

ATRX MEDs are observed in both neuroblastoma and osteosarcoma. Therefore, we undertook a detailed analysis of the *ATRX* MEDs identified in 77 neuroblastoma tumors and two cell lines and six osteosarcoma tumors and one cell line. For neuroblastoma, 29 unique MEDs were identified (Figures 3A and S1 and Table S1). Most commonly a deletion of exon 2–10 (28 of 79, 35.4%, MED#1) was observed, which was predicted to be in-frame (called exon 1–11 IFF in Qadeer et al.) and to produce a mutant protein product.[16] The deletion of exon 2–9 also occurred frequently (11 tumors and two cell lines, MED#2). Although this deletion is predicted to be out-of-frame, on the mRNA level alternative

splicing with skipping of exon 10 was identified, thus resulting in the same mutant protein product as for a deletion of exon 2–10[6]. The third most common deletion consists of exons 3–9 (2–10 IFF, five tumors and one cell line, MED#3), followed by several deletions that only occurred in two patients. The remaining 19 distinct deletions occurred only in a single case (Figure S1A). We also identified one MED that precisely covered the entire *ATRX* gene (Table S1, MED#29). We never observed a tumor with the same MED occurring in homozygosity. Of all deletions, 75% (59 of 79) was shown or predicted to be in-frame and therefore could result in mutant protein production in most of the tumors. The minimal deleted region, the part that is lost in most cases, was exon 8 and 9 (Figure 3B). Upon close examination of all deletions, two distinct subgroups can be identified, namely the ones missing exon 8–9 and cases with smaller deletions that included loss of exon 11–12 (Figures 3A,B, S1, and S2). Interestingly, this is in sharp contrast to the missense mutations that are enriched in the helicase domain, while the deletions mostly involve the N-terminal domains. This suggests a whole other type of functional consequence on the molecular level, as different protein-interacting domains are affected and therefore could lead to different impairments of ATRX's functions.

FIGURE 3 Mutational landscape of recurrent multiexon deletions (MEDs) in neuroblastoma. (A) Overview of all recurrent MEDs in our dataset that occur in more than one patient. Numbers (#1–#9) indicate the unique MED identifier, and the number before every deletion indicates the number of patients with that specific aberration. Symbols after every deletion indicate whether the deletion is predicted to be in-frame (Y) or not (N). Y* is predicted to be in-frame because of exon skipping of exon 10. (B) Exon landscape plot showing the presence of each exon in all neuroblastoma MEDs in our dataset, including the deletions displayed in Figure S1

(A)

(B)
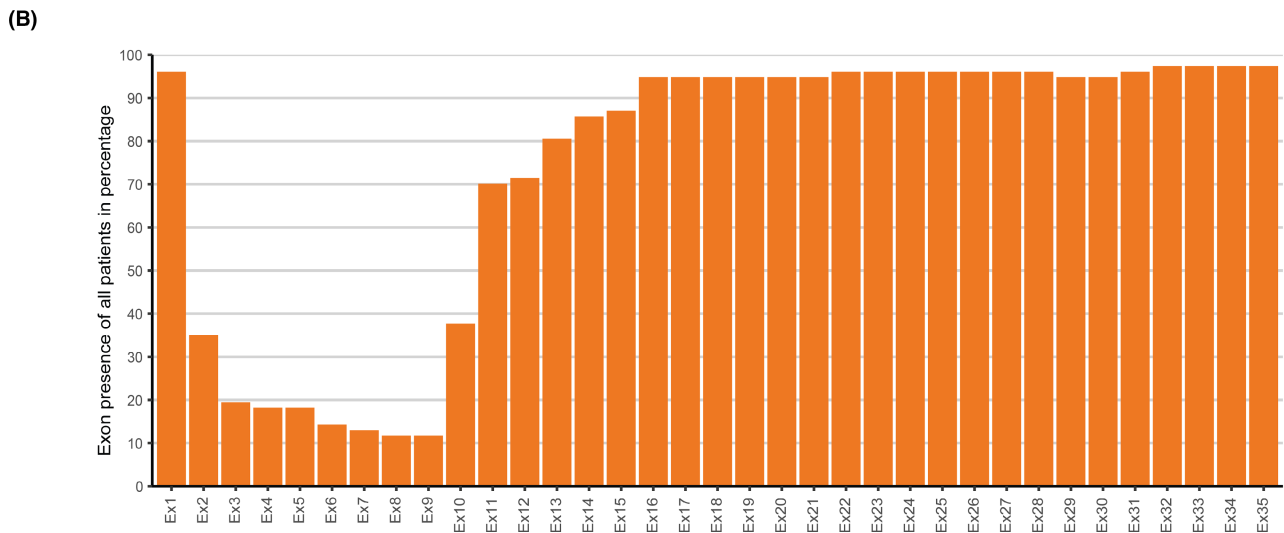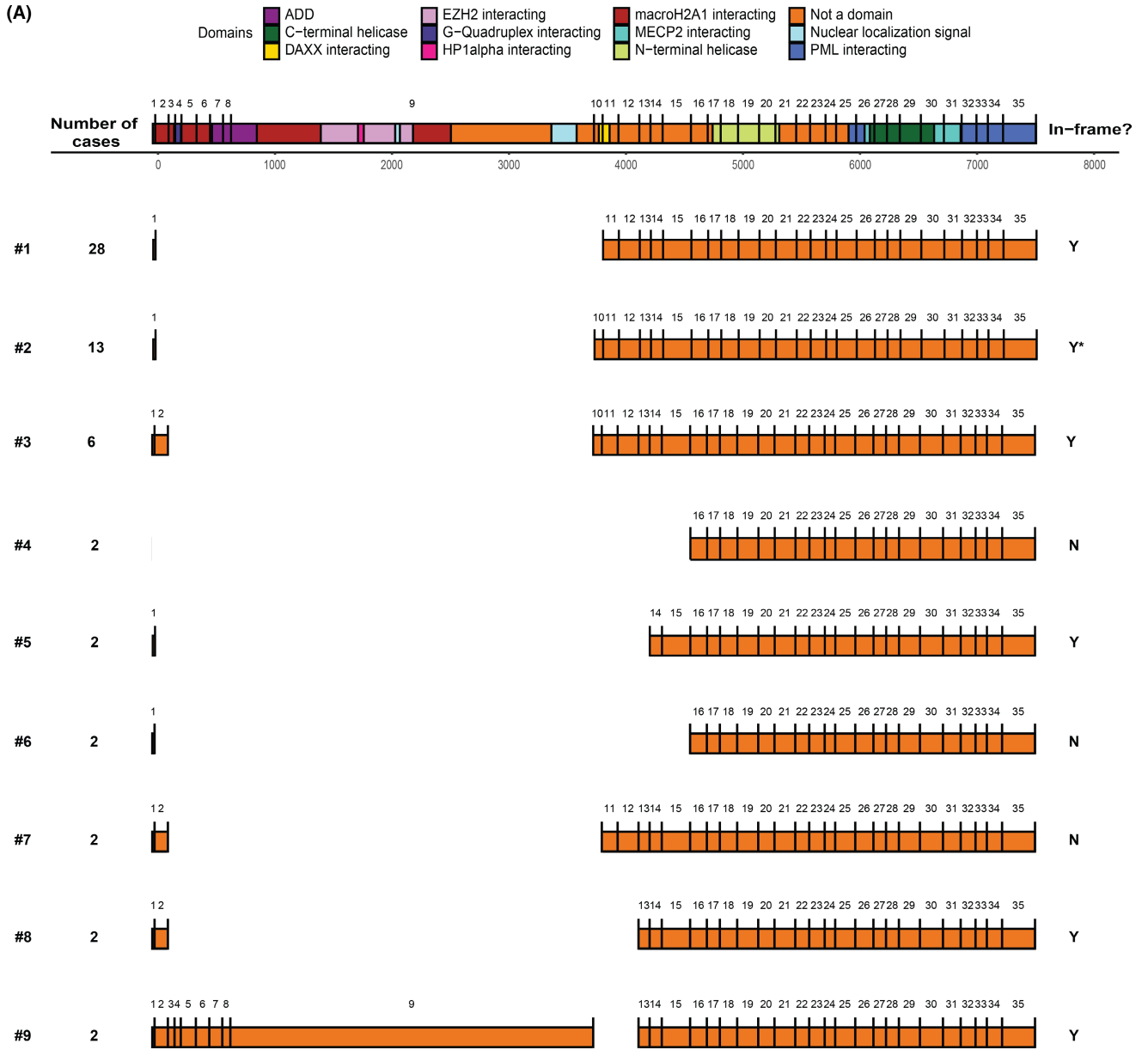
Subsequently, we also wanted to examine deletions in other pediatric cancers and found that large deletions only occurred in osteosarcoma and B-ALL (Table S1). However, in B-ALL there were three out-of-frame MEDs, all different from the MEDs in neuroblastoma. In osteosarcoma there were seven reported MEDs, of which two were also reported in neuroblastoma, namely the predicted out-of-frame deletion of exon 2–15 (MED#6) and the predicted IFD of 2–13 (MED#5), of which the latter was the only recurrent aberration for osteosarcoma. The remaining four deletions were all predicted to be out-of-frame. In summary, we determined that MEDs were mostly observed in neuroblastoma and rarely occur in other pediatric cancers. Additionally, we found that 75% of *ATRX* deletions in neuroblastoma are predicted to be in-frame and that there are two patterns of deletions, namely those lacking exons 8–9 or exons 11–12.

3.4 | Correlations of *ATRX* mutation subtypes with clinical characteristics

There were several reports that showed that patients with neuroblastoma carrying ATRX aberrations are diagnosed at an older age.[20,61,62] We wanted to investigate this for all three mutation subtypes separately. As a reference cohort for this analysis we used the cohort of Pugh et al.[28] This cohort consisted of 240 stage 4 tumors, of which 24 had an *ATRX* aberration. After removing these samples, which were included in our ATRX subtypes, we had a reference cohort of 216 tumors. We took this cohort as it consisted of only stage 4 tumors, which was also almost the case for the *ATRX* tumors collected from published reports. Additionally, it was one of the very few studies reporting many of the SCAs observed in neuroblastoma. As none of the collected patients with *ATRX* aberrations belonged to a single cohort, the use of this reference cohort might introduce a systematic bias. We observed that there was a difference in the distribution of the age at diagnosis between the reference cohort and all three *ATRX* mutation subtypes (Mann–Whitney *U*-test, deletion $p = 2.6e-13$, nonsense $p = 7.7e-05$ and missense $p = 0.00053$) (Figure 4A). Interestingly, this difference was found for all three subtypes, even for the patients with missense aberrations for which we had relatively few data points.

To confirm the different age distribution, we separated the patients with and without *ATRX* aberrations into three categories (18–60 months, 5–12 years, and >12 years) according to their age at diagnosis (Figure 4B; no patients were younger than 18 months of age in this cohort). We chose a cut-off of 5 years as 90% of neuroblastoma patients are younger than 5 years old at diagnosis,[63] and a cut-off of 12 years because of the start of puberty. Eighty-three percent of patients in the reference cohort (*ATRX* WT) were 5 years or younger, whereas less than 42% of patients were in this age group for the three *ATRX* mutation types. Compared to the reference cohort, the *ATRX* subtypes had a significantly different contribution of each age category (Table S3), except for the 5–12 years age category, where there was no significant difference between the reference

cohort and the missense and nonsense subtype (Fisher's exact test, $p_{adj} = 0.88$ and $p_{adj} = 0.12$, respectively). This could, however, be explained by the relatively low number of patients for these subtypes. Overall, this indicates that all three *ATRX* subtypes occurred more frequently in patients diagnosed at an older age, but still two-fifth of the patients were diagnosed below 5 years of age.

The *ATRX* gene is located on the X chromosome and therefore *ATRX* aberrations in neuroblastoma might affect male individuals more often than female individuals. To investigate this, we compared the male : female ratio in all three mutation types to the ratio reported in a large neuroblastoma cohort, which is 1.1:1.[64] Interestingly, a higher male : female ratio than expected, with a ratio of 1.77:1 (Figure 4C), was detected in patients with *ATRX* MEDs (binominal test, $p = 0.040$). In contrast, the male : female ratio was not found to be skewed when *ATRX* was affected by missense or nonsense mutations (Figure 4C; binominal tests; $p = 0.79$ and $p = 0.64$, respectively). Our reference cohort had a relatively high male : female ratio of 1.6:1. However, male : female ratios of 1.2:1,[65] 1.3:1,[66] and 1.65:1[67] have also been reported in neuroblastoma cohorts and when we used these ratios the deletion subgroup was no longer significantly different. However, the ratio of 1.1:1 that we used for our analysis was derived from a large cohort across different continents and therefore seems more reliable.[64] Thus, our analysis suggests that *ATRX* deletions, but not other *ATRX* aberrations, might occur more often in male than female patients. Nevertheless, more thorough investigation is necessary in the future.

Patients with *ATRX* aberrant neuroblastoma are known to have poor event-free and overall survival.[5,61] Here we assessed the event-free and overall survival for each mutation type separately (Figure 4D). For event-free survival, there was no difference between the three groups (Gehan–Breslow–Wilcoxon test, $p = 0.098$). However, a significantly better overall survival (Gehan–Breslow–Wilcoxon test, $p = 0.022$) of patients with missense mutations was observed as compared to the other two mutation types. Thus, patients with *ATRX* missense mutations might have a slightly better prognosis.

3.5 | Neuroblastomas with *ATRX* deletions more often have 11q deletion and/or 2p gain

Segmental chromosomal aberrations are common in neuroblastoma and several of these recurrent aberrations are associated with a poor prognosis, such as 17q gain, 11q deletion, 2p gain, and 1p deletion.[62] To investigate the co-occurrence of these SCAs in the three subgroups of *ATRX* aberrant tumors and one cell line (CHLA-90), we plotted their frequencies compared to the reference cohort. This comparison showed no significant differences in the proportion of tumors with 17q gain, or 1p or 3p deletion (Figure 5 and Table S4). In contrast, 11q deletion ($p_{adj} = 1.2694e-10$) and 2p gain ($p_{adj} = 0.021$) more often occurred in tumors with *ATRX* deletions compared to the other two *ATRX* mutation types or the reference cohort (Figure 5). This was most striking for the 11q deletion, as almost 90% of the patients with an *ATRX* deletion carried this SCA. 11q deletion and MNA
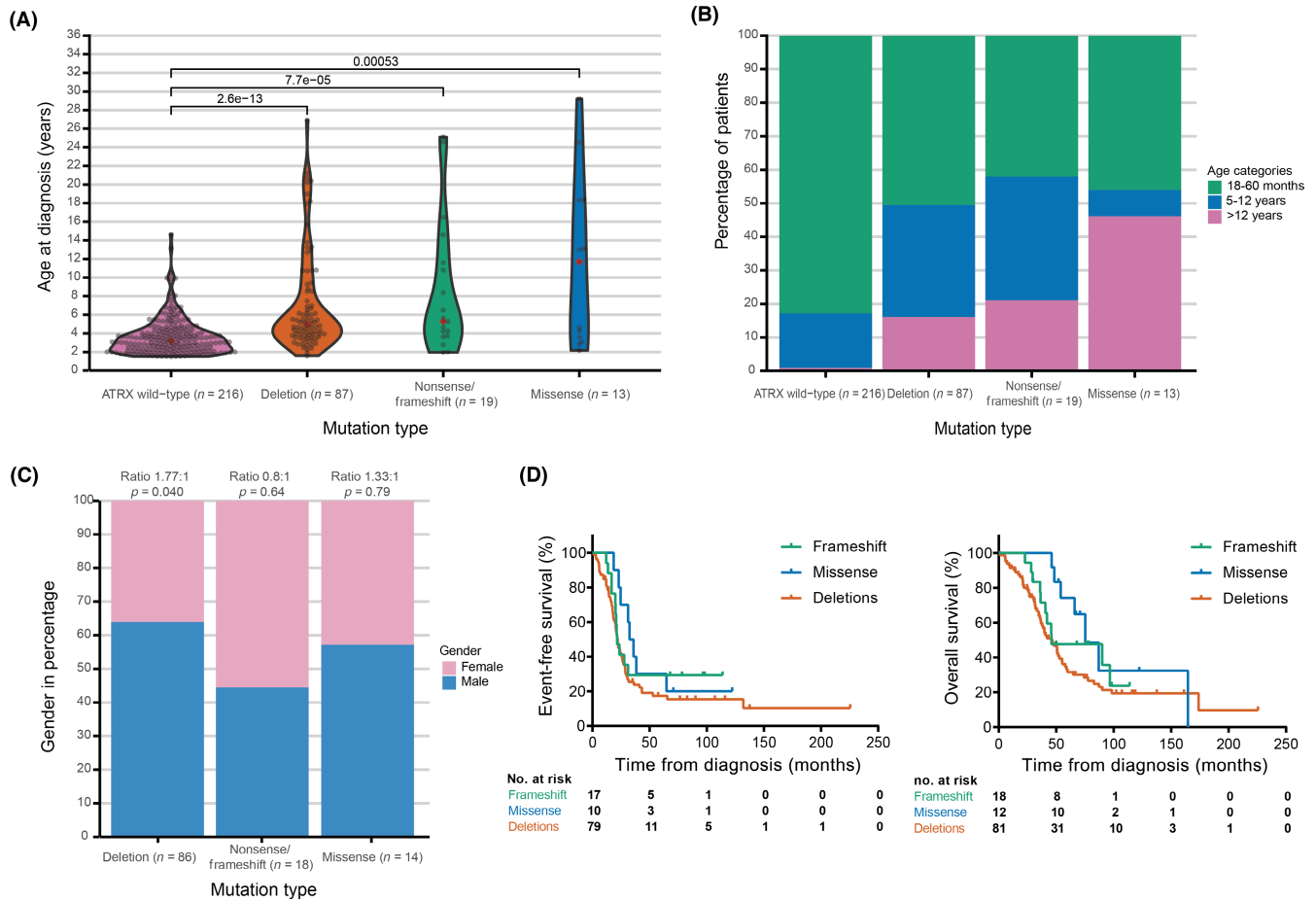
FIGURE 4 Correlations between *ATRX* mutation subtypes with clinical characteristics in neuroblastoma. (A) Violin plot showing the distribution of age at diagnosis (years) for each of the mutation types. Red diamonds show medians: ATRX WT, 3.18 years; Deletion, 5 years; Nonsense/frameshift, 5.28 years; Missense, 11.7 years; *p* values (Mann–Whitney *U*-test) are displayed. (B) Percentage of patients belonging to each age category for every mutation type. Fisher's exact tests were carried out for each age category (18–60 months, 5–12 years, and >12 years) to compare the mutation types. Both *p* values and adjusted *p* values can be found in Table S3. (C) Distribution of gender in percentages within the *ATRX* aberrant subtypes. (D) Kaplan–Meier curves of event-free survival (left) and overall survival (right) of the *ATRX* aberrant subtypes

are almost mutually exclusive,[68] and the reference cohort contains approximately 36% MNA tumors; in contrast, MNA is rarely reported for *ATRX* aberrant tumors. Therefore, the observed enrichment of 11q deletions in tumors with *ATRX* deletions might result from a bias caused by MNA. To exclude this possibility, we carried out the analysis again after excluding all MNA tumors from the reference cohort. Even though the percentage of patients with 11q deletion increased in the reference cohort, there was still a significant difference for both 11q deletion ($p_{adj} = 0.0004$) and 2p gain ($p_{adj} = 0.02$) between the *ATRX* deleted tumors and the reference cohort (Figure S3 and Table S5). Thus, this was a strong indication that the co-occurrence of 11q deletion occurred more frequently in the deletion subtype than in the other two *ATRX* mutation types.

Additionally, it is important to consider the status of the X chromosome, as *ATRX* is localized on it. In male individuals, a single *ATRX* aberration will abolish WT *ATRX* expression, however, female individuals normally have two alleles. Even though the high prevalence of in-frame MEDs might suggest a gain-of-function/dominant effect, this

still must be experimentally confirmed. The X chromosomal status was reported for 24 of 47 female patients in our study. For one female patient (Table S1, AMC802T), both alleles contained an *ATRX* aberration; for another female patient (PASTKW), two *ATRX* aberrations were also reported and potentially inactivated both alleles (could also be on the same allele). From the remaining 22 female patients, seven tumors lost one X chromosome and therefore the detected *ATRX* aberrations will abolish WT *ATRX* expression. However, we also observed two girls and one boy in which the tumor acquired one X chromosome. Thus, not always both *ATRX* alleles are no affected by aberrations in all tumors; nevertheless, ALT has been reported in at least some of these tumors, suggesting a potential dominant effect.

Finally, we also investigated the co-occurrence of MNA, *ALK*, and *TP53* mutations with *ATRX* aberrations. *MYC-N* amplification was reported in only 4 of 121 *ATRX* aberrant tumors (two nonsense, one missense, and one MED tumor) (Table S1), which could be attributed to heterogeneous MNA as previously described.[69] *ALK* status and *TP53* status were only reported in a subset of the *ATRX* aberrant
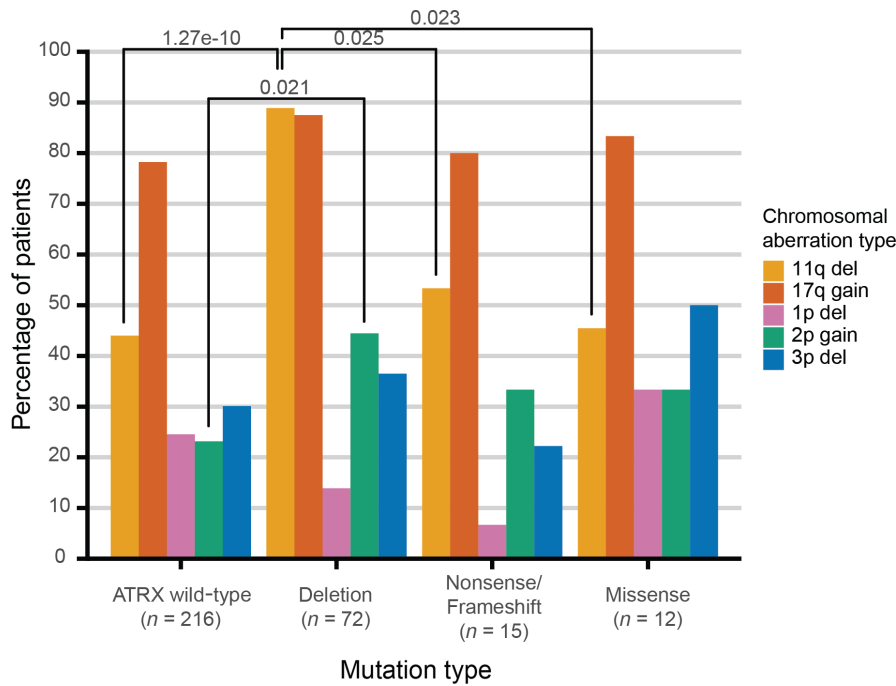
FIGURE 5 Co-occurrence of *ATRX* aberrations with segmental chromosomal aberrations (SCAs). Percentage of patients with neuroblastoma-specific SCAs for the *ATRX* mutation types and the *ATRX* WT reference cohort (including *MYC-N* amplified tumors). Only significant *p* values after multiple testing correction are displayed (Fisher's exact tests). del, deletion

tumors (71 and 46 tumors, respectively; Table S1). *ALK* mutations occurred in 14 of these tumors, including all *ATRX* mutation types. Interestingly, *TP53* mutations were only identified in two samples, which are the cell lines SK-N-MM and CHLA-90 carrying an *ATRX* IFD. Thus, neuroblastoma-typical SCAs and mutations co-occur with *ATRX* aberrations and specifically 11q deletions are more frequently observed within tumors with *ATRX* deletions.

3.6 | All types of *ATRX* aberrations are associated with ALT

ATRX aberrations are often associated with ALT and neuroblastomas with *ATRX* aberrations are more likely to have ALT than *ATRX* WT tumors.[61] To investigate the frequency of ALT in the *ATRX* aberrant neuroblastomas from this study, we checked whether their ALT status was reported. The ALT status was assessed in a total of 44 tumors with *ATRX* aberrations, and a total of 43 samples were confirmed to have ALT (Table S1). The only ALT-negative tumor contained a missense mutation at L407F, which was predicted as neutral and damaging by the PROVEAN and SIFT algorithms, respectively. The negative ALT status of this tumor might support a neutral role of this mutation in the tumor biology, although it could also be a false negative sample. Overall, these results confirm the strong association between the co-occurrence of ALT with *ATRX* aberrations in neuroblastoma.

4 | DISCUSSION

The aim of this study was to characterize the mutational spectrum of *ATRX* aberrations in neuroblastoma tumors and to present an overview of the accompanying tumor and patient characteristics.

In this manner we identified a mutational hotspot region for *ATRX* missense mutations within the helicase domain. Interestingly, we determined that nearly 70% of the *ATRX* aberrations in neuroblastoma are MEDs, compared to only approximately 10% in other pediatric cancers. Additionally, we found that 75% of these MEDs are predicted or known to be in-frame and could result in protein production. Our separate comparison of the three mutational subtypes with *ATRX* WT tumors revealed that 11q deletion only co-occurs more frequently with *ATRX* deletions. Finally, we identified that, even though there is a higher frequency of *ATRX* aberrations in patients diagnosed at an older age, still approximately 40% of patients are younger than 5 years of age at diagnosis.

Although *ATRX* aberrations are frequently point mutations in most pediatric cancers, they represent a minority of the *ATRX* aberrations in neuroblastoma. We showed that in neuroblastoma the *ATRX* nonsense mutations were equally distributed across the *ATRX* gene. The effect of *ATRX* nonsense mutations in neuroblastoma has not been extensively studied. It is likely that these mutations lead to nonsense-mediated mRNA decay or to an unstable protein prone to degradation. Unlike the random distribution of *ATRX* nonsense mutations, we identified the helicase domain as a missense mutational hotspot region in neuroblastoma. It is currently unknown whether missense mutations lead to reduced or absence of ATRX protein production in neuroblastoma. Interestingly, in patients with ATR-X syndrome, a disease characterized by moderate intellectual disability and mild symptoms of α-thalassemia, most of the missense mutations result in an unstable protein, more prone to degradation and resulting in reduced protein abundance. As the helicase domain is required for the DNA translocation activity of ATRX,[70] defects in this domain could lead to disruption of histone variant H3.3 incorporation or other remodeling processes, such as removal of G-quadruplexes or R-loops. Therefore, *ATRX* point mutation models could illuminate the precise effects of the patient-specific aberrations.

Multiexon deletions of the *ATRX* gene are almost exclusively present in neuroblastoma and rare in other pediatric malignancies investigated in this study. We determined that 75% of these deletions are known or predicted to be in-frame in neuroblastoma. Several studies have reported that these in-frame ATRX deletions still result in protein expression.[16,61] This high percentage of IFDs indicates that there could be a gain-of-function effect of these aberrations. This effect might have been identified by Qadeer et al., who postulated that ATRX IFFs are still able to localize to chromatin with an alternative binding pattern.[16] We also showed that the deletions can be divided into two subgroups, namely the more frequent larger deletions including exons 8–9, and the less common smaller deletions lacking exons 11–12. These smaller deletions, which so far have not been well studied, could have different molecular effects in neuroblastoma cells as compared to bigger deletions and therefore might need other therapies. The low frequency of these smaller deletions could be explained by these being much harder to detect in genome sequencing data, which is the most often used technique to identify such aberrations. Additionally, we determined that 25% of the deletions are predicted to be out-of-frame; nonetheless some could still produce in-frame transcripts by using exon skipping, which has been reported for the second most common deletion of exon 2–9 (MED#2).[6]

In our study we determined that there was a trend towards a gender bias in the group of patients with *ATRX* deletions, however, this was heavily dependent on the used reference ratio and therefore should be investigated on a larger cohort. In addition, it is often not reported whether female patients have one or two affected *ATRX* alleles. This is also complicated by whole X chromosome losses or gains and random XCI that occurs during each cell division in female cells. Interestingly, ATRX is involved in the maintenance of XCI[71] and both *ATRX* alleles might be transcribed in female patients. Support for this comes from a study that generated a conditional heterozygous KO of *ATRX* in female cells of the 8- to 16-cell state of developing mice that resulted in abrogation of XCI in extraembryonic tissues.[72] Perturbed XCI has already been observed in several cancers including breast and colorectal cancer.[73] Thus, also for neuroblastoma, it will be important to determine whether the *ATRX* aberrant allele is transcribed in all or a proportion of the tumor cells, which might be relevant for treatment strategies.

It has previously been described that patients with neuroblastoma diagnosed at an older age more often had *ATRX* aberrations than younger patients.[20,61,62] Our study confirmed this observation for each of the three mutation types independently in a cohort of 119 patients with *ATRX* aberrations. However, we also identified that almost 40% of the patients were younger than 5 years of age, which is much more frequent than described in earlier publications that contained fewer patients.[20,61] Furthermore, patients with *ATRX* aberrations have been reported to have a very poor overall and event-free survival.[5,61] We observed this as well for the three mutation types regarding event-free survival, but patients with a missense

mutation had a better overall survival. An explanation could be that some of the observed missense mutations might be passenger variants that do not affect function, therefore patients might have been misclassified as ATRX aberrant.

In neuroblastoma there are few recurring mutations, but almost all high-risk patients carry SCAs, of which many have been associated with poor overall survival.[62,74] Here we showed that almost 90% of the tumors with an *ATRX* deletion also have a 11q deletion, which was more frequent than in the reference cohort. The association of *ATRX* aberrations and 11q deletions has very recently been reported,[61,62] but in our study this is only the case for tumors with *ATRX* deletions and not for *ATRX* point mutations. The minimal region of overlap lost in 11q deletions contains a lot of genes involved in apoptosis and in cell cycle progression.[68]*ATRX* aberrations are known to result in defective replication restart and concomitantly lead to increased DNA damage.[75] Therefore, it could be evolutionary advantageous to acquire an 11q deletion when *ATRX* is aberrant to reduce the levels of DNA damage-induced apoptosis, although it is also possible that the 11q deletion is already present before the *ATRX* deletion occurs and therefore provides the appropriate molecular environment. Why this co-occurrence is more frequent for *ATRX* deletions than for point mutations and the order of the mutational events needs further investigation. We also show that *ATRX* deletions and 2p gain often co-occur. Interestingly, in our dataset there were four tumors in which both an *ATRX* aberration and MNA were present. However, MNA and *ATRX* aberrations are reported to be clinically mutually exclusive[5,62] and elevated MYCN expression and ATRX loss were shown to be synthetically lethal in mouse models and cell lines.[61] Nevertheless, for some tumors, heterogeneity of MNA and *ATRX* aberrations has been observed,[69,76] which could be a potential explanation for the patients with both aberrations in our dataset. Another possibility is that, in some instances, MNA might not result in high MYCN protein expression. Finally, *TP53* mutations are very rare in our dataset, and are only present in two cell lines, where they might have been introduced in vitro. In contrast to glioma, where there is a strong co-occurrence of *TP53* and *ATRX* aberrations,[77] we did not find such an association for neuroblastoma.

The major limitation of this study is the lack of data of both patient and tumor characteristics for many of the categories that we investigated, such as overall survival and SCA status. We are especially lacking a lot of information for the less frequently observed SCAs (2p gain and 3p del) and for the mutational status of *ALK* and *TP53*. This lack of information might have influenced our outcomes and therefore our results should be re-evaluated in the future. Most importantly, the X chromosomal or *ATRX* copy number status are frequently not reported, even though this might be important information for female patients. In the future, it would be of great value if more complete and more standardized clinical and tumor data would be reported and if sequencing data would be publicly available.

In conclusion, this study shows that *ATRX* MEDs are the most frequent type of *ATRX* aberrations in neuroblastoma and that 75% of these are expected to produce in-frame deleted protein products with a potential gain of function. We also identified a more frequent co-occurrence of 11q deletions and *ATRX* deletions in neuroblastoma tumors. Overall, our study highlights that the different types of *ATRX* aberrations have distinct characteristics. It emphasizes the need for in vitro and in vivo models of these different aberrations, which might cause unique molecular phenotypes and require distinct treatments.

DISCLOSURE
The authors declare that there are no competing interests.

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REFERENCES
1. Maris JM. Recent advances in neuroblastoma. *N Engl J Med*. 2010;362(23):2202-2211.
2. Matthay KK, Maris JM, Schleiermacher G, et al. Neuroblastoma. *Nat Rev Dis Prim*. 2016;2.
3. Jansky S, Sharma AK, Körber V, et al. Single-cell transcriptomic analyses provide insights into the developmental origins of neuroblastoma. *Nat Genet*. 2021;53(5):683-693.
4. Cohn SL, Pearson ADJ, London WB, et al. The International Neuroblastoma Risk Group (INRG) classification system: an INRG task force report. *J Clin Oncol*. 2009;27(2):289-297.
5. Valentijn LJ, Koster J, Zwijnenburg DA, et al. TERT rearrangements are frequent in neuroblastoma and identify aggressive tumors. *Nat Genet*. 2015;47(12):1411-1414.
6. Ackermann S, Cartolano M, Hero B, et al. A mechanistic classification of clinical phenotypes in neuroblastoma. *Science*. 2018;362(6419):1165-1170.
7. Hoang SM, O'Sullivan RJ. Alternative lengthening of telomeres: building bridges to connect chromosome ends. *Trends Cancer*. 2020;6(3):247-260.
8. Lewis PW, Elsaesser SJ, Noh KM, Stadler SC, Allis CD. Daxx is an H3.3-specific histone chaperone and cooperates with ATRX in replication-independent chromatin assembly at telomeres. *Proc Natl Acad Sci U S A*. 2010;107(32):14075-14080.
9. Law MJ, Lower KM, Voon HPJ, et al. ATR-X syndrome protein targets tandem repeats and influences allele-specific expression in a size-dependent manner. *Cell*. 2010;143(3):367-378.
10. Li Y, Syed J, Suzuki Y, et al. Effect of ATRX and G-Quadruplex formation by the VNTR sequence on α-globin gene expression. *ChemBioChem*. 2016;17(10):928-935.
11. Ratnakumar K, Duarte LF, LeRoy G, et al. ATRX-mediated chromatin association of histone variant macroH2A1 regulates α-globin expression. *Genes Dev*. 2012;26(5):433-438.
12. Nguyen DT, Voon HPJ, Xella B, et al. The chromatin remodelling factor ATRX suppresses R-loops in transcribed telomeric repeats. *EMBO Rep*. 2017;18(6):914-928.
13. Valle-García D, Qadeer ZA, McHugh DS, et al. ATRX binds to atypical chromatin domains at the 3′ exons of zinc finger genes to preserve H3K9me3 enrichment. *Epigenetics*. 2016;11(6):398-414.
14. Gibbons RJ, McDowell TL, Raman S, et al. Mutations in ATRX, encoding a SWI/SNF-like protein, cause diverse changes in the pattern of DNA methylation. *Nat Genet*. 2000;24(4):368-371.
15. Huh MS, Ivanochko D, Hashem LE, et al. Stalled replication forks within heterochromatin require ATRX for protection. *Cell Death Dis*. 2016;7(5):e2220-e2312.
16. Qadeer ZA, Valle-Garcia D, Hasson D, et al. ATRX in-frame fusion neuroblastoma is sensitive to EZH2 inhibition via modulation of neuronal gene signatures. *Cancer Cell*. 2019;36(5):512-527.e9.
17. Abbasi MR, Rifatbegovic F, Brunner C, et al. Impact of disseminated neuroblastoma cells on the identification of the relapse-seeding clone. *Clin Cancer Res*. 2017;23(15):4224-4232.
18. Bellini A, Bessoltane-Bentahar N, Bhalshankar J, et al. Study of chromatin remodeling genes implicates SMARCA4 as a putative player in oncogenesis in neuroblastoma. *Int J Cancer*. 2019;145(10):2781-2791.
19. Brunner C, Brunner-Herglotz B, Ziegler A, et al. Tumor touch imprints as source for whole genome analysis of neuroblastoma tumors. *PLoS One*. 2016;11(8):1-15.
20. Cheung N-KV. Association of age at diagnosis and genetic mutations in patients with neuroblastoma. *JAMA*. 2012;307(10):1062-1071.
21. Fransson S, Östensson M, Djos A, et al. Estimation of copy number aberrations: comparison of exome sequencing data with SNP microarrays identifies homozygous deletions of 19q13.2 and CIC in neuroblastoma. *Int J Oncol*. 2016;48:1103-1116.
22. Koneru B, Lopez G, Farooqi A, et al. Telomere maintenance mechanisms define clinical outcome in high-risk neuroblastoma. *Cancer Res*. 2020;80(12):2663-2675.
23. Kurihara S, Hiyama E, Onitake Y, Yamaoka E, Hiyama K. Clinical features of ATRX or DAXX mutated neuroblastoma. *J Pediatr Surg*. 2014;49(12):1835-1838.
24. Lasorsa VA, Formicola D, Pignataro P, et al. Exome and deep sequencing of clinically aggressive neuroblastoma reveal somatic mutations that affect key pathways involved in cancer progression. *Oncotarget*. 2016;7(16):21840-21852.
25. Ma X, Liu Y, Liu Y, et al. Pan-cancer genome and transcriptome analyses of 1,699 paediatric leukaemias and solid tumours. *Nature*. 2018;555(7696):371-376.
26. Mazzocco K, Defferrari R, Sementa AR, et al. Genetic abnormalities in adolescents and young adults with neuroblastoma: a report from the Italian Neuroblastoma Group. *Pediatr Blood Cancer*. 2015;62(10):1725-1732.
27. Molenaar JJ, Koster J, Zwijnenburg DA, et al. Sequencing of neuroblastoma identifies chromothripsis and defects in neuritogenesis genes. *Nature*. 2012;483(7391):589-593.
28. Pugh TJ, Morozova O, Attiyeh EF, et al. The genetic landscape of high-risk neuroblastoma. *Nat Genet*. 2013;45(3):279-284.
29. Suzuki M, Kushner BH, Kramer K, et al. Treatment and outcome of adult-onset neuroblastoma. *Int J Cancer*. 2018;143(5):1249-1258.
30. Wei JS, Johansson P, Chen L, et al. Massively parallel sequencing reveals an accumulation of de novo mutations and an activating mutation of LPAR1 in a patient with metastatic neuroblastoma. *PLoS One*. 2013;8(10):1-13.
31. Zhang J, Walsh MF, Wu G, et al. Germline mutations in predisposition genes in pediatric cancer. *N Engl J Med*. 2015;373(24):2336-2346.
32. Chami R, Marrano P, Teerapakpinyo C, et al. Immunohistochemistry for ATRX can miss ATRX mutations: lessons from neuroblastoma. *Am J Surg Pathol*. 2019;43(9):1203-1211.
33. Dagg RA, Pickett HA, Neumann AA, et al. Extensive proliferation of human cancer cells with ever-shorter telomeres. *Cell Rep*. 2017;19(12):2544-2556.

34. Ambros IM, Brunner C, Abbasi R, Frech C, Ambros PF. Ultra-high density SNParray in neuroblastoma molecular diagnostics. *Front Oncol.* 2014;4:202. doi:10.3389/fonc.2014.00202

35. Gröbner SN, Worst BC, Weischenfeldt J, et al. The landscape of genomic alterations across childhood cancers. *Nature.* 2018;555(7696):321-327.

36. Pinto EM, Chen X, Easton J, et al. Genomic landscape of paediatric adrenocortical tumours. *Nat Commun.* 2015;6(1):1-10.

37. Lindqvist CM, Lundmark A, Nordlund J, et al. Deep targeted sequencing in pediatric acute lymphoblastic leukemia unveils distinct mutational patterns between genetic subtypes and novel relapse-associated genes. *Oncotarget.* 2016;7(39):64071-64088.

38. Zhang J, McCastlain K, Yoshihara H, et al. Deregulation of DUX4 and ERG in acute lymphoblastic leukemia. *Nat Genet.* 2016;48(12):1481-1489.

39. D'Angelo F, Ceccarelli M, Tala, et al. The molecular landscape of glioma in patients with Neurofibromatosis 1. *Nat Med.* 2019;25(1):176-187.

40. Buczkowicz P, Hoeman C, Rakopoulos P, et al. Genomic analysis of diffuse intrinsic pontine gliomas identifies three molecular subgroups and recurrent activating ACVR1 mutations. *Nat Genet.* 2014;46(5):451-456.

41. Schwartzentruber J, Korshunov A, Liu XY, et al. Driver mutations in histone H3.3 and chromatin remodelling genes in paediatric glioblastoma. *Nature.* 2012;482(7384):226-231.

42. Hoffman LM, DeWire M, Ryall S, et al. Spatial genomic heterogeneity in diffuse intrinsic pontine and midline high-grade glioma: implications for diagnostic biopsy and targeted therapeutics. *Acta Neuropathol Commun.* 2016;4(1):1-8.

43. Phillips JJ, Gong H, Chen K, et al. The genetic landscape of anaplastic pleomorphic xanthoastrocytoma. *Brain Pathol.* 2019;29(1):85-96.

44. Salloum R, McConechy MK, Mikael LG, et al. Characterizing temporal genomic heterogeneity in pediatric high-grade gliomas. *Acta Neuropathol Commun.* 2017;5(1):1-12.

45. Sorge C, Li R, Singh S, et al. Complete durable response of a pediatric anaplastic oligodendroglioma to temozolomide alone: case report and review of literature. *Pediatr Blood Cancer.* 2017;64(12):1-4.

46. Wu G, Diaz AK, Paugh BS, et al. The genomic landscape of diffuse intrinsic pontine glioma and pediatric non-brainstem high-grade glioma. *Nat Genet.* 2014;46(5):444-450.

47. Zhang J, Wu G, Miller CP, et al. Whole-genome sequencing identifies genetic alterations in pediatric low-grade gliomas. *Nat Genet.* 2013;45(6):602-612.

48. Ali NM, Niada S, Brini AT, et al. Genomic and transcriptomic characterisation of undifferentiated pleomorphic sarcoma of bone. *J Pathol.* 2019;247(2):166-176.

49. Chen X, Bahrami A, Pappo A, et al. Recurrent somatic structural variations contribute to tumorigenesis in pediatric osteosarcoma. *Cell Rep.* 2014;7(1):104-112.

50. Ji J, Quindipan C, Parham D, et al. Inherited germline ATRX mutation in two brothers with ATR-X syndrome and osteosarcoma. *Am J Med Genet Part A.* 2017;173(5):1390-1395.

51. Kovac M, Blattmann C, Ribi S, et al. Exome sequencing of osteosarcoma reveals mutation signatures reminiscent of BRCA deficiency. *Nat Commun.* 2015;6:1-9.

52. Masliah-Planchon J, Lévy D, Héron D, et al. Does ATRX germline variation predispose to osteosarcoma? Three additional cases of osteosarcoma in two ATR-X syndrome patients. *Eur J Hum Genet.* 2018;26:1217-1221.

53. Smolle MA, Heitzer E, Geigl JB, et al. A novel mutation in ATRX associated with intellectual disability, syndromic features, and osteosarcoma. *Pediatr Blood Cancer.* 2017;64(10):1-2.

54. Akan P, Alexeyenko A, Costea PI, et al. Comprehensive analysis of the genome transcriptome and proteome landscapes of three tumor cell lines. *Genome Med.* 2012;4(11):1-13.

55. Huether R, Dong L, Chen X, et al. The landscape of somatic mutations in epigenetic regulators across 1,000 paediatric cancer genomes. *Nat Commun.* 2014;5:1-7.

56. Li B, Brady SW, Ma X, et al. Therapy-induced mutations drive the genomic landscape of relapsed acute lymphoblastic leukemia. *Blood.* 2020;135(1):41-55.

57. Dyer MA, Qadeer ZA, Valle-Garcia D, Bernstein E. ATRX and DAXX: mechanisms and mutations. *Cold Spring Harb Perspect Med.* 2017;7(3):a026567.

58. Choi Y, Chan AP. PROVEAN web server: a tool to predict the functional effect of amino acid substitutions and indels. *Bioinformatics.* 2015;31(16):2745-2747.

59. Team R Development Core. *A Language and Environment for Statistical Computing.* R Found Stat Comput; 2020;2. https://www.R-project.org

60. Wickham H. *ggplot2: Elegant Graphics for Data Analysis.* Springer-Verlag; 2016.

61. Zeineldin M, Federico S, Chen X, et al. MYCN amplification and ATRX mutations are incompatible in neuroblastoma. *Nat Commun.* 2020;11(1):1-20.

62. Brady SW, Liu Y, Ma X, et al. Pan-neuroblastoma analysis reveals age- and signature-associated driver alterations. *Nat Commun.* 2020;11(1):1-13.

63. Howlader N, Noone AM, Krapcho M, et al. *SEER Cancer Statistics Review, 1975–2009 (Vintage 2009 Populations).* National Cancer Institute.

64. Georgakis MK, Dessypris N, Baka M, et al. Neuroblastoma among children in Southern and Eastern European cancer registries: variations in incidence and temporal trends compared to US. *Int J Cancer.* 2018;142(10):1977-1985.

65. Tas ML, Reedijk AMJ, Karim-Kos HE, et al. Neuroblastoma between 1990 and 2014 in the Netherlands: increased incidence and improved survival of high-risk neuroblastoma. *Eur J Cancer.* 2020;124:47-55.

66. Hale G, Cula MJ, Blatt J. Impact of gender on the natural history of neuroblastoma. *Pediatr Hematol Oncol.* 1994;11(1):91-97.

67. Moussa E, Fawzy M, Younis A, et al. Combined treatment strategy and outcome of high risk neuroblastoma: experience of the Children's Cancer Hospital-Egypt. *J Cancer Ther.* 2013;4(9):1435-1442.

68. Mlakar V, Jurkovic Mlakar S, Lopez G, et al. 11q deletion in neuroblastoma: a review of biological and clinical implications. *Mol Cancer.* 2017;16:1-12.

69. Berbegall AP, Bogen D, Pötschger U, et al. Heterogeneous MYCN amplification in neuroblastoma: a SIOP Europe Neuroblastoma Study. *Br J Cancer.* 2018;118(11):1502-1512.

70. Mitson M, Kelley LA, Sternberg MJE, Higgs DR, Gibbons RJ. Functional significance of mutations in the Snf2 domain of ATRX. *Hum Mol Genet.* 2011;20(13):2603-2610.

71. Baumann C, De La Fuente R. ATRX marks the inactive X chromosome (Xi) in somatic cells and during imprinted X chromosome inactivation in trophoblast stem cells. *Chromosoma.* 2009;118(2):209-222.

72. Garrick D, Sharpe JA, Arkell R, et al. Loss of Atrx affects trophoblast development and the pattern of X-inactivation in extraembryonic tissues. *PLoS Genet.* 2006;2(4):438-450.

73. Spatz A, Borg C, Feunteun J. X-chromosome genetics and human cancer. *Nat Rev Cancer.* 2004;4(8):617-629.

74. Szewczyk K, Wieczorek A, Młynarski W, et al. Unfavorable outcome of neuroblastoma in patients with 2p gain. *Front Oncol.* 2019;9:1018.

75. Leung JWC, Ghosal G, Wang W, et al. Alpha thalassemia/mental retardation syndrome X-linked gene product ATRX is required for proper replication restart and cellular resistance to replication stress. *J Biol Chem.* 2013;288(9):6342-6350.

76. Hartlieb SA, Sieverling L, Nadler-Holly M, et al. Alternative lengthening of telomeres in childhood neuroblastoma from genome to proteome. *Nat Commun.* 2021;12(1):1-18.

77. Liu XY, Gerges N, Korshunov A, et al. Frequent ATRX mutations and loss of expression in adult diffuse astrocytic tumors carrying IDH1/IDH2 and TP53 mutations. *Acta Neuropathol.* 2012;124(5):615-625.

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