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ARTICLE

Analysis of the mutational status of *SIX1/2* and microRNA processing genes in paired primary and relapsed Wilms tumors and association with relapse

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Abstract

Whereas 90% of patients with Wilms tumor (WT) reach cure, approximately half of patients developing a recurrent tumor die of the disease. Therefore, to disclose events leading to recurrence represents a clinical need. To study paired primary/recurrent tumor samples, being aware of the intra-tumoral heterogeneity, might help finding these answers. We previously suggested that mutations in *SIX1* and *DROSHA* underlie WT recurrence. With the aim to better investigate this scenario, we collected 19 paired primary/recurrent tumors and 10 primary tumors from relapsing patients and searched for mutations in the *SIX1/2* genes and microRNA processing genes (miRNAPGs). We found *SIX1* mutation in one case, miRNAPGs mutations in seven cases, and the co-occurrence of *SIX1* and miRNAPG mutations in one case. We could observe that, whereas in primary tumors the mutations could be heterogeneously present, in all cases they were positively selected and homogeneously present in the recurrent disease, as also indicated by a "moderate" and "almost perfect" agreement (according to the Landis and Koch classification criteria) between paired samples. Analysis of *SIX1/2* genes and miRNAPGs in 50 non-relapsing WTs disclosed *SIX2* mutation in one case and miRNAPGs mutations in seven. A borderline statistically significant association was observed between miRNAPGs mutations and the occurrence of relapse (p value: 0.05). These data suggest that *SIX1* and miRNAPGs mutations may provide an advantage during tumor progression to recurrence and can represent oncogenic drivers in WT development.

Introduction

The overall survival of children suffering from Wilms tumor (WT), the most common renal tumor in childhood, is now ~90% [1]. However, half of relapsing patients develop resistance to salvage therapies and die of the disease. Thus, there is the need to understand the molecular features underlying tumor resistance to therapies and recurrence, in order to identify the potential targets to address for an effective treatment. There are growing data indicating that tumors are dynamic diseases: even after malignant transformation, tumors keep on evolving, generating a molecularly heterogeneous mass consisting of distinct clones of cells with different characteristics and levels of sensitivity to therapies. Treatment represents a selective pressure for the tumor, and a heterogeneous tumor has major chances to possess a resistant cellular clone or to evolve one [2]. Hence, we believe that a comprehensive understanding of tumor heterogeneity is key to develop effective and durable therapeutic strategies. Recently, the intra-tumor genetic heterogeneity of primary WTs has been documented for a number of genomic anomalies, among which 1q copy number (CN) gain, *MYCN* activation, *TP53, SIX1*, and

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DROSHA mutations [3–6]. Two studies identified mutations in *SIX1*, *SIX2* and in the microRNA processing genes (miRNAPGs) *DICER1*, *DROSHA*, and *DGCR8*, reporting data that suggested these anomalies as potentially associated with a poor prognosis when found in primary tumors [7, 8]. In particular, *SIX1/SIX2* and *DROSHA/DGCR8* mutations were shown to underlie high-risk blastemal-type WTs in patients treated with preoperative chemotherapy according to the protocols of the Sociètè Internationale d'Oncologie Pediatrique (SIOP), while the co-occurrence of *SIX1/SIX2* and *DROSHA/DGCR8* mutations was associated with a worse outcome in patients with favorable histology tumors managed with primary nephrectomy according to the protocols of the Children's Oncology Group [7, 8].

The genetic anomalies affecting recurrent diseases, and in particular the events leading the primary tumor to relapse, are scarcely investigated in WT due to the difficulty in recruiting tumor samples at relapse, to match with the corresponding primary tumor samples. Two previous studies presented whole-genome data comparing primary tumor and matched corresponding relapse sample. The first, on ten such paired samples, demonstrated CN gains at chromosomes 5p, 8p12, 15q, 16p, and 20q, and CN losses of 11q and 17p as events acquired in two recurrent tumors [9]. The second, on eight paired primary/relapsed tumors, showed chromosomal anomalies at 1q, 3, 16q in WT recurrences, and the co-occurrence of *SIX1* and *DROSHA* mutations in the recurrence in three patients. Interestingly, the investigation of the primary tumors of these three patients displayed the heterogeneous presence of *DROSHA* mutations in all cases, and the presence of a *SIX1* mutation in only one case, thus suggesting that these mutations were involved in the process of tumor recurrence [6].

To better investigate the involvement of *SIX1/SIX2* and miRNAPGs in WT recurrence, we collected primary tumors from children suffering from tumor recurrence, together with the corresponding recurrences, when available. For each primary tumor, and when possible for available relapses, multiple samples were investigated. The primary endpoint of our study was to investigate in paired primary/recurrent tumor samples whether the pattern of mutations in the *SIX1/SIX2* and miRNAPGs possibly disclosed in the primary disease was consistently maintained in the recurrent tumors, and the secondary endpoint was to assess if mutations in these genes were associated with relapse.

Materials and methods

Patients

In 2016, the centers of the Associazione Italiana Ematologia Oncologia Pediatrica (AIEOP) that had registered WT patients who relapsed in the AIEOP-2003 and SIOP-2001 protocols were asked to participate in this study. General inclusion into the study was based on: (1) diagnosis of WT relapse and (2) availability of multiple formalin-fixed paraffin-embedded (FFPE) samples of the tumor at diagnosis (primary tumor sample). For the study primary endpoint, eligibility was also based on the availability of the corresponding paired sample at recurrence (recurrent tumor sample). Overall, 19 paired primary/recurrent tumors were centralized (representing Cohort A1). For statistical considerations, an additional eight paired samples from primary and recurrent tumors, described in our previous analysis were also included (Cohort A2) [6]. Study sample size was not formally estimated also in consideration of the rarity of the investigated disease. Accordingly, *SIX1/SIX2* and miRNAPGs mutations were evaluated in all available primary and recurrent tumors. To elaborate on the secondary endpoint of the study, two additional cohorts were collected. Cohort B included ten WTs from patients who relapsed and for whom only samples from the primary tumor were available, due to different reasons (e.g., scarcity of material at recurrence, recurrent tumor not surgically resected). Cohort C was represented by 50 WTs from non-relapsing patients (treated at the Fondazione IRCCS Istituto Nazionale dei Tumori, Milan (INT)), for which multiple FFPE blocks from the primary tumor were available.

The study was approved by the Ethics Committees of Fondazione IRCCS Istituto Nazionale dei Tumori, Milan (AIEOP protocol) and of Bambino Gesù Pediatric Hospital and IRCCS, Rome (SIOP protocol), and specific informed consent to the use of biological samples for the aim of the study was obtained from the parents or the guardians of all patients. Researchers were not blinded to patients' Cohort allocation. Clinico-pathological characteristics of relapsing patients are depicted in Supplementary Table S1.

Selection of tumor tissue

All available pathological material of the collected cases was centrally reviewed by an expert pediatric pathologist. Classification along the AIEOP TW2003 and SIOP protocols was applied. For each case, multiple FFPE blocks from different areas of the tumor mass were selected, so to include all the different histological components present in the tumor (i.e., epithelium, blastema, and stroma). When present, anaplastic component was also included. Five slides of 5 µm were cut for each selected block and macrodissection was applied when needed, to obtain at least 90% of viable tumor cells for each block.

Molecular analyses

DNA was extracted using the GeneRead^{TM} DNA FFPE Kit (Qiagen, Milan, Italy). *SIX1* (CCDS9748) and *SIX2*

(CCDS1822) p.Q177R hotspot mutations, *DGCR8* (CCDS13773) p.E518K hotspot mutations, *DROSHA* (ENST00000511367) exons 23, 24, 29, 30, 31, covering RNAse IIIa and RNAse IIIb domains mutations, *DICER1* (ENST00000343455) exons 23, 24, 25, covering all mutations identified in the RNAse IIIb domain, were investigated by Sanger sequencing. Supplementary Table S2 describes PCR and sequencing conditions. Every mutation identified was confirmed by independent PCR and sequencing.

Statistical analyses

Pivotal variables generation

Statistical analysis was performed by considering as pivotal variables SIX (*SIX1/SIX2)* and miRNAPG mutations. Due to the different number of tissue samples analyzed for each patient, the percentage of mutated blocks (PMB_{score}) relative to the total number of those analyzed was computed for each of the considered pivotal variables. All blocks in which a mutation was observed were considered as mutated, irrespective if the mutation appeared to be in homozygosity, in balanced heterozygosity, clonal (i.e., clearly visible, but present in <100% of cells), or subclonal (i.e., present in small cell subpopulations and thus barely visible). Each tumor (primary and recurrent) was categorized, for each pivotal variable, according to two-class and three-class classification criteria. In the first categorization (dichotomized-PMB_{scores}) the two considered classes were: (1) wild type (i.e., all blocks wild type, mutated blocks = 0%) and (2) mutated (i.e., at least one block mutated, mutated blocks > 0%). In the second categorization (categorized-PMB_{scores}) the three classes were: (1) wild type (i.e., all blocks wild type, mutated blocks = 0%); (2) intermediately mutated (i.e., 0% < mutated blocks ≤ 99%); and (3) fully mutated (i.e., 100% of mutated blocks). In addition, to evaluate the co-occurrence of mutations, a joint variable, named SIX + miRNAPGs, was created and dichotomized as follows: (1) with co-occurring mutations and (2) without co-occurring mutations.

Concordance analysis

To analyze the pattern of agreement between the mutational status in the paired tumor samples (primary vs. recurrent), the Cohen's kappa statistic (kc) and its 95% confidence interval (CI) [10] were used when the dichotomized-PMB_{score} was considered. Each kc value was interpreted in a qualitative manner on the basis of the Landis and Koch classification criteria [11]. Prevalence-adjusted and bias-adjusted kappa (PABAK) was also computed to account for imbalance of prevalence [12]. The same analysis was performed by considering the SIX + miRNAPGs joint variable. In addition, to better explore the agreement between the primary and the recurrent samples the weighted kappa statistic (kw) [10] was estimated by starting from the categorized-PMB_{score}. The kw statistic is the most widely accepted measure of agreement if, as in this case, the data in question arise from an ordinal scale. As previously reported [13], the observed values of kw values were considered satisfactory if equal to or >0.80. This analysis was performed on the group of 27 patients with the availability of primary and recurrent samples (named as Subset 1: Cohorts A1 + A2).

Association analysis

To evaluate the pattern of association between the mutational status in the primary tumor and the occurrence of relapse, we considered the group of patients (Subset 2) who either relapsed within 36 months ($n = 35$, including 19 cases from Cohort A1, six cases from Cohort A2 and ten cases from Cohort B) or remained relapse free for at least 36 months after diagnosis ($n = 45$ from Cohort C). Comparison of mutational status of each of the considered pivotal variable in relapsed and relapse-free patients was performed by resorting the Fisher exact test.

For explorative purpose only, in the subgroup of 68 patients recruited and followed at Fondazione IRCCS Istituto Nazionale dei Tumori, Milan (Subset 3, including 18 relapsing cases—7 from Cohort A1, 8 from Cohort A2, and 3 from Cohort B—and the 50 non-relapsing cases of Cohort C), the patterns of Relapse-Free Survival (RFS) were estimated by using the Kaplan–Meier method [14], and the survival curves were compared using log-rank tests. RFS was calculated as the time from diagnosis to the first relapse or last follow-up for relapse-free patients. Given the small number of events only the miRNAPG dichotomized-PMB_{score} was considered in this analysis. All statistical analyses were performed with the SAS software (version 9.4; SAS Institute Inc. Cary, NC) by adopting a significance alpha level of 0.05 (two sided).

Results

SIX1/2 and miRNAPG mutations in paired samples from primary and recurrent tumors

Molecular analyses

Sequence analysis of 19 paired tumor samples (Cohort A1) disclosed co-occurring *SIX1* and *DROSHA* mutations in one case and miRNAPGs mutations in five cases (Supplementary Table S1 and Supplementary Fig. 1). In Patient No. 1 (affected with bilateral tumor), the *DROSHA* p.E1147K mutation was present in all three examined samples macrodissected from two FFPE blocks of the left kidney (two with

stromal and one with epithelial components), whereas was absent in the single block of the right kidney (showing blastemal and epithelial components). Both examined blocks of the relapse developed on the left kidney remnants showed posttreatment blastemal component and the presence of the *DROSHA* mutation (Supplementary Fig. 2). In Patient No. 5 (pre-treated WT), four different areas from three FFPE blocks were selected. Only one sample with blastemal component carried both the *SIX1* p.Q177R and *DROSHA* p.E1147K mutation. In the other three areas (two characterized by epithelial component and one by blastemal–epithelial morphology), one of which macrodissected ca. 3 mm apart from the double mutated sample, only the *SIX1* mutation was barely detectable as a subclonal event. When examining the untreated lung nodule (characterized by blastemal component), all the four FFPE blocks showed both the *SIX1* and *DROSHA* mutations. In Patient No. 8 (pre-treated bifocal WT), one primary nodule was completely necrotic at surgery. Thus, only three different FFPE blocks (two with mixed blastemal–stromal components and one with triphasic morphology, all with diffuse anaplasia) from the second nodule could be inspected. In one block, the *DGCR8* p.E518K mutation was in homozygosity, while in the other two, the wild-type allele was present only in clonal or subclonal measure. In the single block from the pre-treated lung recurrence (characterized by blastemal component), the mutated allele was the most represented allele. In Patient No. 10 (pre-treated WT), three blocks (all with blastemal–epithelial components) were examined, and in the two showing also nuclear unrest or anaplasia the *DGCR8* p.E518K mutation was identified. In two blocks obtained from the pre-treated abdominal recurrence and the aortic metastasis at first recurrence (both with blastemal–epithelial components with anaplasia), the mutation was present in almost complete homozygosity, and in the pre-treated peritoneal mass at second recurrence (with blastemal–epithelial components) in complete homozygosity. In Patient No. 11 (pre-treated WT) in both the investigated blocks (with triphasic morphology), the *DICER1* p.D1709G mutation was present. The same mutation was also evident in the single block of the lung metastasis (with triphasic morphology). Finally, in Patient No. 17 (pre-treated WT) in the two blocks (with blastemal component) that were examined, the *DGCR8* p.E518K mutation was present, but in one only at clonal level; in the untreated lung recurrence (with blastemal component) the same mutation was found.

Concordance analysis

Considering the dichotomized-PMB_{score} on the 27 patients of Subset 1, we found a "moderate" and "almost perfect" agreement (according to the Landis and Koch classification criteria) between paired samples with kc values of 0.63 (95% CI: 0.17–1.00) and 1.00, when considering the SIX and miRNAPG mutations, respectively (Table 1). According to the Landis and Koch classification criteria, a "moderate" level of agreement was also obtained when the SIX + miRNAPGs joint variable was considered (Table 2). The "moderate" level of agreement observed for SIX mutations and the SIX + miRNAPGs joint variable was due to presence of two cases (Patients No. 51 and No. 262, from Cohort A2) that were mutated for *SIX1* in the recurrence but not in the paired primary tumor, whereas no cases showed a different mutational profile for miRNAPGs alone between the primary and relapsed tumor. By considering the PABAK values, a higher level of agreement was observed for the SIX mutations (PABAK: 0.85, 95% CI: 0.65-1.00). Pursuing the analysis by considering the categorized-PMB_{score} (Table 3), the observed level of concordance (kw: 0.63, 95% CI: 0.17–1.00) for SIX mutations did not differ from that observed for the dichotomized-PMB_{score}, as no samples had an intermediately mutated profile in both the primary and recurrent tissue. On the contrary, for the miRNAPG mutations, we observed six cases (Patients Nos. 1, 5, 10 from Cohort A1; Nos. 51, 74, 262 from Cohort A2) with an intermediately mutated profile in the primary tumor, but a fully mutated profile in the recurrent tumor, leading to a kw value of 0.84 (95% CI: 0.74–0.94).

Table 1 Concordance table of the dichotomized-PMB_{score} for SIX and miRNAPGs mutations.

Recurrent Primary	Wild type	Mutated	Total
SIX			
Wild type	23	2	25
Mutated	0	2	2
Total	23	4	27
miRNAPGs			
Wild type	18	0	18
Mutated	0	9	9
Total	18	9	27

SIX1/2 and miRNAPG mutations in primary WTs from relapsing and non-relapsing patients

Sequence analysis of ten primary tumors from patients which eventually relapsed, but whose recurrent tumors were not available for molecular analyses (Cohort B) disclosed one *SIX1* and two miRNAPGs mutations (Supplementary Table S1 and Supplementary Fig. 1). In Patient No. 22 (untreated WT), the *SIX1* p.Q177R mutation was found in all three examined FFPE blocks (two with blastemal component and one with blastemal–epithelial components). In Patient No. 23 (untreated WT), we investigated three

Table 2 Concordance table of the joint variable SIX + miRNAPGs.

Recurrent / Primary	No co-occurrence mutation	Co-occurrence mutation	Total
No co-occurrence mutation	23	2	25
Co-occurrence mutation	0	2	2
Total	23	4	27

Table 3 Concordance table of the categorized-PMB_{score} for SIX and miRNAPGs mutations.

Recurrent / Primary	Wild type	Intermediate mutation	Full mutation	Total
SIX				
Wild type	23	0	2	25
Intermediate mutation	0	0	0	0
Full mutation	0	0	2	2
Total	23	0	4	27
miRNAPGs				
Wild type	18	0	0	18
Intermediate mutation	0	0	6	6
Full mutation	0	0	3	3
Total	18	0	9	27

different FFPE blocks (all with blastemal–stromal components) all of which carried the *DROSHA* p.E1147K mutation, although in two cases the mutation was clonal. In Patient No. 26 (untreated WT), of whom three FFPE blocks (all with triphasic morphology) were selected, a *DICER1* p.D1713V mutation was found in all the investigated samples and, more precisely, in homozygosity in one sample, as the most represented allele in another sample, and in heterozygosity in the third sample.

Sequence analysis in 50 primary WT samples of non-relapsing patients (Cohort C) showed a *SIX2* mutation in one case and miRNAPGs mutations in seven cases (Supplementary Fig. 3). In particular, in Patient No. NR6 (untreated WT) a *DICER1* p.G1809R mutation was detected in one of the two examined FFPE blocks (both with stromal component); Patient No. NR11 (pre-treated WT) showed a *DROSHA* p.E993K mutation in heterozygosity in one block (with stromal–epithelial components) and as subclonal event in the other (with triphasic morphology). Patient No. NR16 (untreated WT) had a *DROSHA* p.E1147K mutation in one out of three FFPE blocks (all with triphasic morphology); Patient No. NR20 (untreated WT) showed an almost homozygous *DGCR8* p.E518K mutation in all three examined FFPE blocks (two with blastemal–stromal components and one with blastemal component). Patient No. NR22 (untreated WT) showed a *DROSHA* p.D1151G mutation in all three examined FFPE blocks (two with triphasic morphology and one with blastemal–stromal components); Patient No. NR46 (pre-treated WT) displayed a *DROSHA* p.D1151A mutation in all three FFPE blocks (all with triphasic morphology). Patient No. NR55 (untreated WT) displayed a *DGCR8* p. E518K mutation in all three FFPE blocks (two with triphasic morphology—in one of which the mutation was in homozygosity—and one with blastemal component), and Patient No. NR42 (untreated WT) showed a *SIX2* p.Q177R mutation in one of three FFPE blocks (all with triphasic morphology).

Association analysis

By considering Subset 2, including patients from all cohorts, with the exception of those that relapsed after 36 months and those that did not relapse, but with a follow-up of <36 months, a borderline statistically significant association was observed between miRNAPG mutations and the occurrence of relapse (Fisher Exact p value: 0.05). No statistically significant associations were found for SIX gene mutations (Fisher Exact p value: 0.314) or for SIX + miRNAPGs joint variable (Fisher Exact p value: 0.188). Figure 1 depicts the frequency of relapsed or relapse-free patients according to the mutational profile. Finally, by looking at 68 INT patients of Subset 3 (median follow-up: 79 months; interquartile range: 54–95 months), no statistical significance was obtained between patients stratified according to the miRNAPGs dichotomized-PMB_{score} (log-rank p value: 0.54), although a better prognosis was observed for patients with a wild-type profile. Figure 2 shows the Kaplan–Meyer curves for the miRNAPGs dichotomized-PMB_{score}.

Discussion

Tumor heterogeneity is a well-known phenomenon in different adult neoplasias, in which the disease typically develops after long periods of time before being clinically diagnosed [2]. Intra-tumor heterogeneity has been poorly investigated in pediatric solid tumors. These tumors carry a lower number of mutational changes and have a shorter evolution time before diagnosis, thus they may be expected

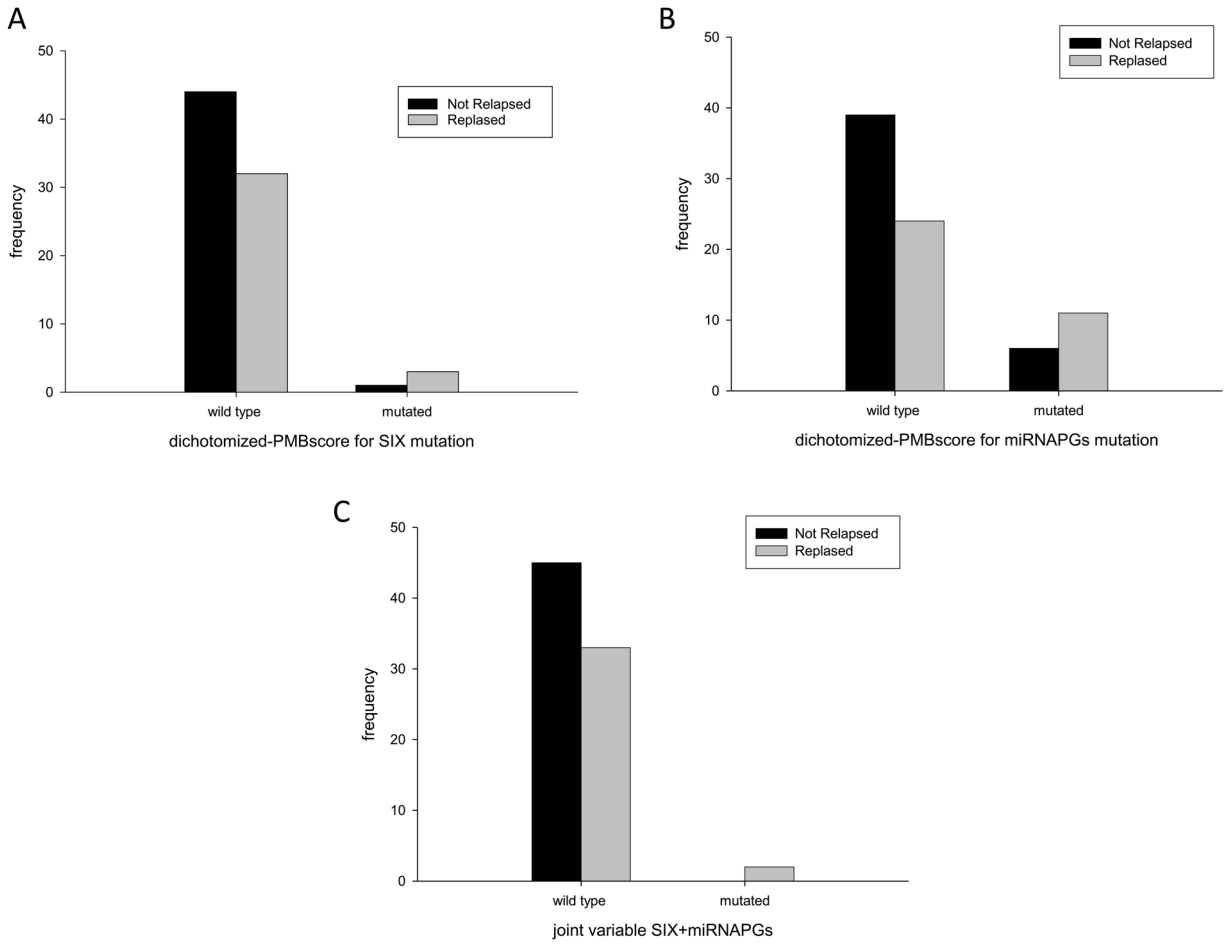

Fig. 1 Frequency distribution of relapsed and relapse-free patients according to the mutational profile. Bar-chart showing the occurrence of relapse according to the dichotomized-PMB_{score} mutational status in the primary tumors of Subset 2 for **A** SIX mutations, **B** miRNAPGs mutations, and **C** the joint variable SIX+miRNAPGs.

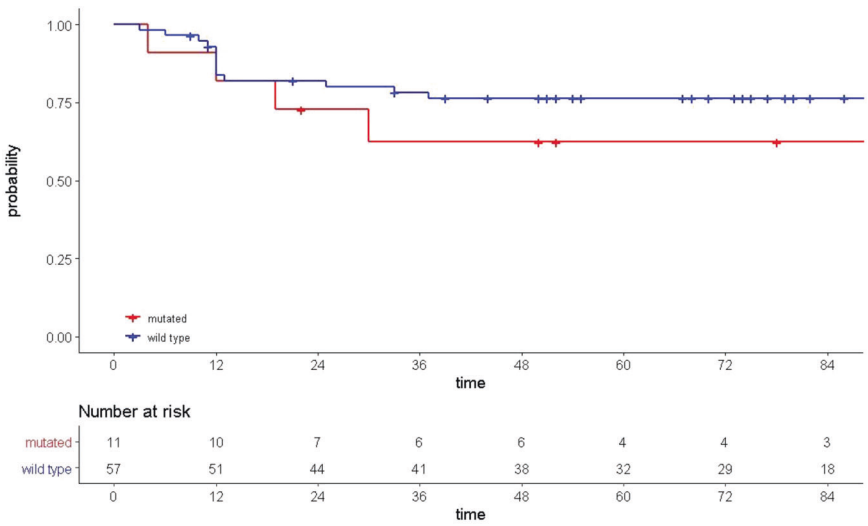

Fig. 2 Kaplan–Meyer curves according to miRNAPGs status. Kaplan–Meyer curves for the miRNAPGs dichotomized-PMB_{score} of the primary tumors of Subset 3.

to show a lower level of complexity and heterogeneity [15]. Noteworthy, to be aware of the heterogeneity issue is of key importance when prognostic histologic or molecular tumor markers are investigated. To date, WT potentially prognostic/prognostic markers, such as chromosome 1q CN gain and combined chromosomes 1p and 16q loss of

heterozygosity [16–19], have been studied relying on a single sampling strategy, assuming that a unique sample is representative of the entire tumor mass. This assumption has recently been questioned [3–6]. In a previous study, performed on eight paired primary/recurrent tumors, we demonstrated that chromosomal anomalies at 1q, 3, 16q and mutations of *SIX1* and *DROSHA* genes underlie WT recurrences [6]. With the aim of improving the level of evidence on the role of *SIX1/2* and miRNAPGs during the evolution and progression of a primary disease to a recurrent tumor, we expanded our previous analysis by collecting additional 19 paired samples. We also implemented a sample strategy analyzing multiple blocks from spatially different areas of the tumor. One major finding was that in all cases in which mutations were observed in the primary disease, although not necessarily in all the blocks, and not necessarily in balanced heterozygosity, the same mutations were invariably present in the corresponding relapsing disease, in all examined blocks and, apparently, in all tumor cells. Intriguingly, in at least one case (Patient No. 10) in which we had the opportunity of investigating multiple recurrences at first and second relapse, all of them displayed the same *DGCR8* mutation, which was present in two out of three blocks from the primary tumor, suggesting that they all originated from a unique mutated cellular clone. In addition, the *DGCR8* mutation was almost reduced to complete homozygosity, thus suggesting the acquisition of an inactivating "second hit" by the recurrent disease. Therefore, the combined analysis of paired tumor tissues, while confirming the previously reported presence of intra-tumor genetic heterogeneity for these mutations [6] allowed us to disclose a positive selection of the mutations identified in the primary tumor during progression toward a relapsed disease. This is further strengthened by the observation of four patients, in whom *SIX1* and *DROSHA* mutations co-occurred in the recurrent disease, including two cases (Nos. 5 and 74) where we observed the positive selection of the primary tumor cell clones bearing both events, and two cases (Nos. 51 and 262) where the *SIX1* mutation was not detected in the primary disease. Taken together, our observations strongly support the notion that tumor cells bearing anomalies in *SIX1* and miRNAPGs (either as a single or co-occurring event) are positively selected during tumor progression to recurrent disease, indicating that the above anomalies possibly represent driver mutations, in at least a subset of relapsing tumors. Concordance analysis, performed on a total of 27 paired primary/recurrent WTs form this study and our previous study [6], disclosed a "perfect agreement" between primary and recurrent tumor when miRNAPGs mutations were considered, and a "moderate" level of agreement when the SIX and SIX + miRNAPGs joint variable were considered. The "moderate" agreement obtained for the SIX mutations and the co-occurrence of SIX + miRNAPGs mutations is due to the acquisition of SIX mutations in two recurrences. Accordingly, the categorized-PMB_{score} disclosed the shift from an intermediate- to a fully mutated profile when moving from primary to recurrent disease.

To obtain a better estimate of the frequency of *SIX1/2* and miRNAPGs anomalies in primary tumors of relapsing patients, we investigated further ten cases (Cohort B). Within the primary tumors of the 37 relapsing patients overall considered, mutations were globally identified in 12 cases (32.4%), including 1 case (2.7%) with *SIX1* mutation only, 9 cases (24.3%) with miRNAPGs mutations only, and 2 (5.4%) with co-occurrence of *SIX1* and miRNAPGs mutations.

Based on our results, we suggest that mutation screening in multiple FFPE blocks is the most accurate method when looking for *SIX1/2* gene and miRNAPGs mutations, which are often heterogeneously present within the primary WT mass. Indeed, we could have missed by chance miRNAPGs mutations in up to six primary tumors from relapsing patients if investigating only one of the selected tissue blocks. This might explain the difference in miRNAPGs mutation frequency that we observed in comparison to other reports, in which, relying on a single-sample strategy, only < 15% of relapsing tumors displayed miRNAPGs mutation in favorable histology [7] and high-risk blastemal [8] WTs.

To evaluate if mutations in the studied genes were associated with increased risk of relapse, we applied the same multiple sampling approach to a series of 50 cases of non-relapsing WTs, in which we could disclose *SIX2* mutation in one case, miRNAPGs mutations in seven, while the co-occurrence of *SIX1/2* and miRNAPGs anomalies was never observed. Again, the intra-tumoral heterogeneous presence of these anomalies was confirmed. Considering the association analysis on Subset 2, a borderline statistically significant association was observed between miRNAPGs mutations and the occurrence of relapse.

Finally, considering only the miRNAPGs mutational status according to the relapse time in 68 patients homogeneously treated at Fondazione IRCCS Istituto Nazionale dei Tumori, Milan (Subset 3), we observed a better prognosis, although not statistically significant, for patients with a wild-type profile.

To conclude, we confirmed that *SIX1/2* and miRNAPGs mutations can be present in the primary disease only in some cellular clones, not representing the entire bulk of the tumor. These mutated cell populations can thus arise during primary tumor growth, but are homogeneously present in relapses, indicating that they are positively selected during tumor progression to a relapsed disease. We suggest that these mutations may provide an advantage during tumor progression to recurrence and, therefore, can represent oncogenic drivers in WT development. Should our

observations be confirmed, *SIX1* and miRNAPGs mutations could account for a relevant fraction of recurring WTs, supporting the usefulness of their screening in primary tumors to identify those patients who may deserve more intense clinical surveillance and/or therapy.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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