Brief Report

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Analysis of microsatellite instability (MSI) in pediatric gonadal and extra-gonadal germ cell tumors

Marco Montella[1,§], Maria Elena Errico[2,§], Andrea Ronchi[1], Giuseppa Zannini[1], Vittoria Donofrio[2], Giovanni Savarese[3], Roberto Sirica[3], Francesco Esposito[4], Marco De Martino[4], Alfonso Papparella[5], Renato Franco[1], Paolo Chieffi[6,*], Federica Zito Marino[1]

[1] Pathology Unit, Department of Mental Health and Physic and Preventive Medicine, University of Campania "Luigi Vanvitelli", Naples, Italy;
[2] Pathology Unit Department of Pathology, Santobono-Pausilipon Children's Hospital, Naples, Italy;
[3] AMES, Centro Polidiagnostico Strumentale srl, Casalnuovo, Naples, Italy;
[4] Institute of Experimental Endocrinology and Oncology (IEOS) "G. Salvatore", National Research Council (CNR), Naples, Italy;
[5] Department of Child, Women, General and Specialized Surgery, University of Campania, Naples, Italy;
[6] Department of Precision Medicine, University of Campania "Luigi Vanvitelli", Naples, Italy.

SUMMARY Gonadal and extragonadal pediatric germ cell tumors (GCTs) are rare neoplasms with different clinical behavior. Although surgery and cisplatin-based chemotherapy are resolutive in most cases, some patients do not respond to chemotherapy and have a worse outcome. Microsatellite instability (MSI) was correlated to resistance to chemotherapy and sensitivity to immunotherapy in different neoplasms. A series of 21 pediatric GCTs were tested by immuno-histochemistry and PCR to evaluate MSI status. Next generation sequencing was applied to further evaluate cases with discordant results between immunohistochemistry and PCR. Twenty-one cases of pediatric GCT were included in the series. The mean age ranged between 1 and 10 years. Nine cases were gonadal GCTs and the remaining 12 were extra-gonadal GCTs. By immunohistochemistry, one case showed a deficit of Mismatch repair (MMR) proteins. This case was a 1-year-old children affected by gonadal yolk sac tumor. However, all cases resulted microsatellite stable (MSS) by PCR and NGS. MSI was not detected in our series of pediatric GCTs, as well as the data present in literature about adult patients with GCTs. Molecular techniques could have a role to confirm the MSI status in case of dMMR by immunohistochemistry.

Keywords germ cell tumor, extragonadal germ cell tumor, MSI, pediatric tumors

1. Introduction

Germ cell tumors (GCTs) are a heterogeneous group of neoplasms with different clinical behavior, occurring in gonads (gonadal germ cell tumors, GGCT) or extra-gonadal sites (extragonadal germ cell tumors, EGCT), mainly including the sacrococcygeal area, the mediastinum, and the brain (*1,2*). GCTs occurring in pediatric patients (aged 0–18 years) have some clinical and biological peculiarities, differently from the adult counter-part. Pediatric GCTs account for 3% of all malignancies and most commonly arise in children younger than 15 years, with a slight female predominance (male: female ratio: 0.8:1) (*3*). Extragonadal GCTs are relatively more frequent in pediatric patients rather than in adults. Indeed, EGCTs account for about 50% of GCTs in children and 10% of GCTs in adults. GCTs originate from the primordial multipotential germ cells that migrate along the body midline to the gonadal sites during embryogenesis. EGCTs are supposed as originating from primordial germ cells that failed to migrate to the gonads (*2,4,5*). Histologically, GCTs include undifferentiated forms (seminoma/dysgerminoma), embryonal-like differentiated forms (embryonal carcinoma and teratoma) and extra embryonal-like forms (choriocarcinoma and yolk sac tumor) (*6*).

Pediatric GCTs are an heterogenous group of neoplasms with different clinical behavior. Pure yolk sac tumor, seminoma, or mixture of the two are largely the most common histotypes in children less than 4 years of age, while tumors occurring in patients around the time of puberty up through young adulthood includes the full range of possible histotypes (*7*). Histology-specific analyses among white persons revealed that EGCTs

of the brain, pineal gland and pituitary gland were predominantly seminomas/dysgerminomas (67%, 74%, 78%, respectively). In contrast, EGCTs of the pelvis were predominately non-seminomas/non-dysgerminomas (96%) (*2*). Although the rate of malignant GCTs differs by age and location, generally about 20% of them are malignant (*7*).

Therapy of GCTs includes surgery, radiation therapy and chemotherapy. Although surgery and radiation therapy are sufficient in benign and early-stage malignant cases, cisplatin-based chemotherapy is generally applied to patients with advanced GCTs and targeted therapy currently has a limited role (*8*). Since introduction of platinum-based chemotherapy in the 1980s, survival of GCTs has dramatically improved so that the five-year survival is over than 80% (*8*). In addition, better survival has been observed for gonadal in comparison with extra gonadal tumors (*7*). Although the combination of surgery and cisplatin-based chemotherapy is resolutive in more than 90% of cases, some patients do not longer respond to chemotherapy or have a late relapse (*8*). Thus, the recognition of cases that will not benefit from conventional chemotherapy, and the use of alternative therapies, are currently needed, mainly for cisplatin-based chemotherapy-resistant patients. In this setting, microsatellite instability (MSI) may represent a promising biomarker, as it was established that different tumors carrying the deficit of Mismatch repair (MMR) proteins may present resistance to conventional chemotherapy and sensitivity to immunotherapy (*9-13*). MSI largely depends on the integrity of MMR complex, which is composed of 4 proteins (MLH1, MSH2, MSH6, and PMS2) that cooperatively detect and cut base-pair mismatches to allow correct re-synthetizations of the DNA strand (*14*). Mismatch repair (MMR) proteins are involved in DNA replication to repair errors, such as point mutations. MLH-1, PMS-2, MSH-2 and MSH-6 are the most relevant MMR proteins involved implicated in cancer development (*14*). Microsatellites are non-coding DNA regions of the hu-man genome that, like coding regions, can accumulate mutations in case of deficient MMR (dMMR). In clinical practice, the status of dMMR is detected by immunohistochemistry (IHC) to test the loss of MMR proteins, while MSI status may be directly indagated by sequencing-based methods, including PCR and next generation sequencing (NGS) (*14,15*). Particularly, the loss of one or more MMR proteins could trigger a MSI status. A proficient MMR system corrects the eventual presence of accumulated mutations, while a defective MMR system leads to global instability of both repetitive sequences and coding regions. MSI can be molecularly categorised into two distinct phenotypes: MSI-high (MSI-H) and MSI-low (MSI-L) (*14*). The former is defined as instability in two or more of the five markers in the Bethesda reference panel (BAT-25, BAT-26, D2S123, D5S346 and D17S250) is detected, while the latter is characterized by instability in only one marker (*16*). Recently, FDA approved the application of immunotherapy to any cancer with a defective MMR system and/or MSI-high genotype. The detection of MSI status has become mandatory in clinical practice for some neoplasms like colon cancer, as a strong predictor of efficacy for immunotherapy (*14*). In recent years, the role of MSI to predict the resistance to systemic chemotherapy in adult patients affected by advanced GCTs has been evaluated in some studies. Although controversial results have been reported, data seem to suggest a positive correlation between MSI and chemotherapy-resistance, and immunotherapy-sensitivity in GCTs (*14,17,18*). On the other hand, the role of MSI in pediatric GCTs is largely unknown.

In this study, we aim to assess the frequency of MSI in pediatric patients with gonadal and extragonadal GCTs, in order to identify additional molecular targets to exploit chemoresistant neoplasms or further therapeutic improvement.

2. Materials and Methods

2.1. Specimens

A series of 21 tissue samples from gonadic and extragonadal GCTs diagnosed between 2019 and 2021 at the University of Campania "L. Vanvitelli" and the "AORN Santobono-Pausilipon" Hospital were collected. Inclusion criteria were: *i*) histological diagnosis of GCT (both GGCT and EGCT) was performed; *ii*) biological material was sufficient to perform IHC and molecular tests; *iii*) age of the patient was less than 19 years at the time of the diagnosis. All 21 cases were reviewed by two experienced pathologists according to the current histological WHO classification (*6*). We retrospectively recorded clinical and pathological findings, including age of the patient at initial diagnosis, gender, tumor site and histological type.

2.2. Immunohistochemistry

Immunohistochemistry was performed on 4 μm thick whole sections for each case, using four antibodies directed against MLH1 (M1 Ventana clone ready for use and Optiview kit revelation, Tucson, AZ, USA), MSH2 (clone G219-1129 Ventana ready to use; Optiview kit revelation), MSH6 (clone SP93 Ventana ready to use; Optiview kit revelation), and PMS2 (clone A16-4 Ventana ready to use; Optiview kit revelation) proteins on the BenchMark XT device (Ventana Medical Systems). Adjacent normal tissue from each sample served as positive controls (*19, 20*). MMR protein loss was defined by the absence of IHC staining in the nucleus of tumor cells. Results were evaluated as follows: *i*) proficient MMR (pMMR), cases showing positive staining of all four MMR; *ii*) defective ex-pression of mismatch repair proteins (dMMR), cases carrying the loss of one of

two heterodimers, including MLH1/PMS2 or MSH2/MSH6 loss. We further considered another subset; *iii*) cases harboring the loss of one MMR and/or the patchy expression of one or more MMR (lopaMMR). Two independent and blinded observers carried out immunohistochemical analysis.

2.3. PCR

Serial sections of 6 μm in thickness from formalin-fixed paraffin-embedded matched normal and tumor tissues were routinely stained, and representative normal and tumor regions were identified by microscopic examination. Genomic DNA was isolated from the paraffin-embedded tissues using the QIAamp DNA mini kit (Qiagen, Valencia, CA, USA) following separation of tumor and normal tissue by manual microdissection (*21*). MSI was determined on tumor DNA using the EasyPGX® readyMSI, including the following mononucleotide repeats: BAT25, BAT26, NR21, NR22, NR24, NR27, CAT25 and MONO27. The test was performed according to the manufacturer's instructions. PCR results were evaluated as follows: *i*) microsatellite stable (MSS), cases with none of the markers unstable; *ii*) microsatellite instability-high (MSI-H), tumor with 2 or more unstable markers; *iii*) microsatellite instability-low (MSI-L), cases with only one marker unstable (in these cases new testing was carried out on non-tumor tissue, if available, to define a germinal mutation).

2.4. NGS

Tumor DNA from selected tumors was sequenced using Illumina TruSightTM Oncology 500 (TSO500) for MSI status determination. The library was prepared according to the manufacturer's protocol using a hybrid capture based TruSight Oncology 500 DNA/RNA NextSeq Kit (Illumina, San Diego, CA, USA). During library preparation, enrichment chemistry was optimized to capture nucleic acid targets from FFPE tissues. In the TSO 500 analysis, unique molecular identifiers were used to determine the unique coverage at each position and to reduce the background noise caused by sequencing and deamination artifacts in the FFPE samples. The MSI score was calculated using 130 homopolymer microsatellite loci targeted by the TSO500 panel according to the manufacturer's instructions. The proportion of unstable MSI sites to total assessed MSI sites was re-ported as a sample-level microsatellite score, in which at least 40 sites were required to determine an MSI score. The MSI status was calculated from microsatellite sites for evidence of instability relative to a set of baseline normal samples that are based on information entropy metrics.

2.5. Institutional review board statement

The study was conducted in accordance with the Declaration of Helsinki, and approved by the Institutional Review Board (or Ethics Committee) of University of Campania "L.Vanvitelli" (protocol code 0007953/I, date 04/06/2020).

2.6. Informed consent statement

Our study was done retrospectively conducted on archival bio-logical samples; formal consent was not required.

3. Results and Discussion

The series analysed in our study included 21 cases of pediatric GCT. Nine out of 21 (42.9%) cases were GGCTs, and the remaining 12 (57.1%) were EGCTs. The mean age of patients was 3.6 years (range: 1–10 years). In our series, 9 out of 21 (43%) patients were male and 12 (57%) were female. Particularly, 5 out of 9 (66%) GGCTs were in testis and 4 (44%) cases in ovary. Among the 12 EGCTs, 9 cases (75%) were sacral, 2 cases (17%) were mediastinal and 1 case (8%) was coccygeal. Concerning the histology, the series included 5 Yolk sac tumor (23.8%), 11 terato-mas (52.4%) (9 mature and 2 immature), and 5 mixed GCTs (23.8%), particularly 4 yolk sac tumor and teratoma (80%) and 1 seminoma and teratoma (20%). Clinical and pathological features of the patients are summarized in Table 1.

All 21 cases were tested by IHC. Twenty cases (95%) resulted proficient MMR, while the remaining 1 case resulted dMMR (5%). The latter was a 1-year-old children affected by pure testicular yolk sac tumor. In detail, the dMMR case showed loss of expression of MLH1/PMS2 and MSH6, and low/patchy expression of MSH2, as showed in Figure 1. Clinical and pathological features and MMR status of the GCTs are detailed in Table 2. All GCT cases were adequate for PCR analysis. All 21 cases (100%) were MSS, MSS representative results of the analysis is shown in Figure 2. The NGS analysis was performed on 17 out of 21 cases, unfortunately, 4 cases of our series have not been tested since the quantity and the quality of the DNA were not adequate for this assay. All 17 cases analyzed were MSS by NGS, confirming the PCR results.

As well defined in literature, pediatric GCTs are a heterogeneous group of neoplasms, including both gonadal and extragonadal forms. Incidence of GCTs depends on the age and the sex, as these neoplasms are more frequent in adolescents and young adults (aged 15–19 years) rather than young children (age 0–4 years). In the United States the incidence rate of GCTs in children is 0.4 per 100,000 in boys and 0.6 per 100,000 in girls, while the incidence rate in adolescents and young adults is 11.4 per 100,000 in males but only one per 100,000 in females . Histologically, teratoma is largely the most common histotype in young children,

Table 1. Clinical and pathological features of the patients

Patient	Age (Year)	Sex	Site of Tumor	Histology
1	1	M	Testis (R)	Yolk Sac Tumor
2	1	M	Testis (R)	Yolk Sac Tumor
3	1	M	Mediastinum	Teratoma
4	1	M	Sacrum	Yolk Sac Tumor
5	1	F	Sacrum	Yolk Sac Tumor and Teratoma (Mature)
6	<1	F	Sacrum	Teratoma (Mature)
7	2	F	Sacrum	Yolk Sac Tumor + Teratoma (Mature)
8	1	F	Coccyx	Teratoma (Mature)
9	1	F	Sacrum	Yolk Sac Tumor
10	2	F	Sacrum	Teratoma (Mature)
11	10	F	Ovary (R)	Yolk Sac Tumor + Teratoma (Immature)
12	9	M	Testis (L)	Seminoma + Teratoma
13	<1	M	Sacrum	Teratoma
14	1	M	Testis (R)	Teratoma
15	6	F	Ovary (L)	Teratoma (Mature)
16	<1	M	Sacrum	Teratoma
17	1	M	Testis (L)	Yolk Sac Tumor
18	7	F	Ovary (R)	Teratoma (Immature)
19	8	F	Ovary (L)	Yolk Sac Tumor + Teratoma (Mature)
20	8	F	Mediastinum	Teratoma (Mature)
21	<1	F	Sacrum	Teratoma (Immature)

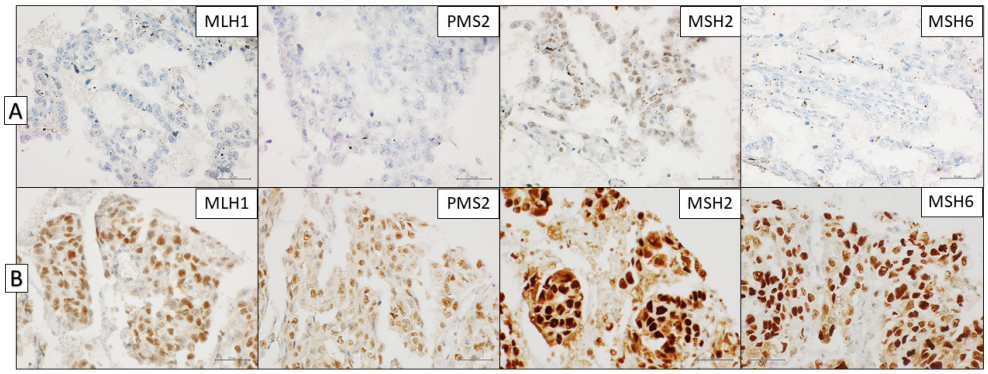

Figure 1. Immunohistochemical evaluation of MSI. (A) Loss of expression of MLH1, PMS2 and MSH6 in tumor cells with positive internal control (DAB coloration, original magnification 40x, scale bar 50μm); heterogeneous expression of MSH2 in tumor cells (DAB coloration, original magnification 40x, scale bar 50μm); **(B)** Representative case with intact expression of MLH1, PMS2, MSH2 and MSH6 (DAB coloration, original magnification 40x, scale bar 50μm).

followed by pure yolk sac tumors. In these patients, loss of chromosomes 1p, 4, 6q and gain of chromosome 1q are the most frequent cytogenetic alterations at this age, and the sacrococcygeal is the most frequent extragonadal location (*22*). In adolescents and young adults, the most common histotypes include teratoma, seminoma/dysgerminoma and mixed GCTs with a higher proportion of embryonal carcinoma and choriocarcinoma (*22*). In these patients, the most common extragonadal location is represented by the mediastinum, and isochromosome 12p (i12p) is the most common cytogenetic alteration (*22*). The clinical behavior of pediatric GCTs is variable and largely depends on histology, and sex and age of the patients. Male adolescents affected by GGCTs showed significantly worse event-free survival (EFS) than children or adults (60% *vs.* 87% and 80%, respectively) in a single-institution study (*23*). Overall, patients with advanced disease achieve a 5-year-overall survival of more than 70%, while patients with non-seminoma histology have a poorer prognosis and 5-year-overall survival of 50% (*24*). Fifteen percent of all patients will develop refractory disease, representing a challenge in the clinical management (*25*).

Although surgery may be curative in benign and early-stage malignant GCTs, advanced-stage GCTs need systemic therapy, which is mainly constituted by cisplatin-based regimens. Moreover, despite the chemo-sensitivity of GCTs, about 10-15% of cases show a resistant phenotype, responsible of tumor relapses and poor prognosis (*26*). The identification of a biomarker able to predict the chemoresistant phenotype should be important to choose the most correct therapy. In this setting, MSI could represent a promising biomarker, as it has been related to platinum-based therapy resistance and immunotherapy sensitivity in other malignant neoplasms (*14*). Two mechanisms could explain the correlation between MSI and treatment resistance. First, MSI may render cells prone to secondary mutations,

Table 2. Clinical and pathological features and MMR status of the GCTs

PATIENT	MLH1	PMS2	MSH2	MSH6	RESULTS
1	Negative	Negative	Patchy	Negative	MSI
2	Positive	Positive	Positive	Positive	MSS
3	Positive	Positive	Positive	Positive	MSS
4	Positive	Positive	Positive	Positive	MSS
5	Positive	Positive	Positive	Positive	MSS
6	Positive	Positive	Positive	Positive	MSS
7	Positive	Positive	Positive	Positive	MSS
8	Positive	Positive	Positive	Positive	MSS
9	Positive	Positive	Positive	Positive	MSS
10	Positive	Positive	Positive	Positive	MSS
11	Positive	Positive	Positive	Positive	MSS
12	Positive	Positive	Positive	Positive	MSS
13	Positive	Positive	Positive	Positive	MSS
14	Positive	Positive	Positive	Positive	MSS
15	Positive	Positive	Positive	Positive	MSS
16	Positive	Positive	Positive	Positive	MSS
17	Positive	Positive	Positive	Positive	MSS
18	Positive	Positive	Positive	Positive	MSS
19	Positive	Positive	Positive	Positive	MSS
20	Positive	Positive	Positive	Positive	MSS
21	Positive	Positive	Positive	Positive	MSS

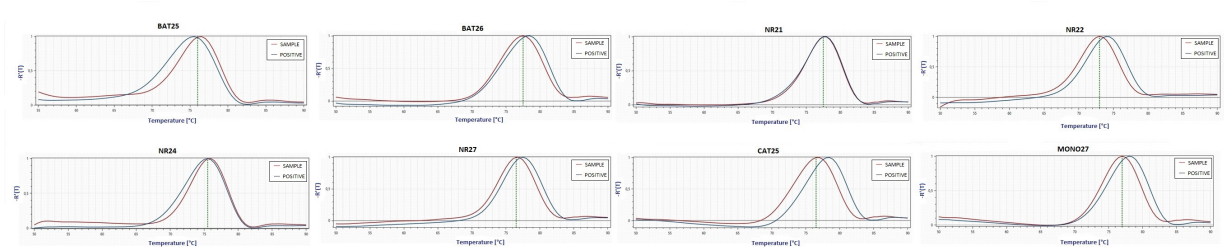

Figure 2. MSS-PCR results. Stability of BAT25, BAT26, NR21, NR22, NR24, NR27, CAT25 and MONO27 (red lines indicate samples while blue lines indicate MSS controls).

leading to resistance. Alternatively, MMR may induce S-phase cell cycle arrest and induction of apoptosis (*27*). Moreover, germ cells and GCTs are characterized by low constitutive activation of the DNA damage response machinery and respond to DNA damage with apoptosis rather than cell cycle arrest (*28*).

We performed literature research using the most diffuse database of scientific report and, to the best of our knowledge, this is the first study indagating MSI status in pediatric GCTs. MSI was indagated by immunohistochemistry, PCR and NGS, in 21 cases of pediatric (less than 10 years old patients) GCTs, including 5 Yolk sac tumor (23.8%) 11 teratomas (52.4%) and 5 mixed GCTs (23.8%). One case resulted dMMR by immunohistochemistry, showing loss of expression of MLH1/PMS2 and MSH6, and low/patchy expression of MSH2. This case was a pure yolk sac tumor occurring in the testis of a 1-year-old children. However, the MSI status was not confirmed by molecular studies, including both PCR and NGS. All cases resulted MSS by PCR. Interestingly, our series confirmed the leading role of molecular studies to define MSI status, suggesting a false-positive result of IHC (*15*).

The potential role of MSI in GCTs is largely unknow, as it was indagated in few studies, limited to adult GCTs. Devouassoux-Shisheboran *et al.* indagated the immunohistochemical expression of hMLH1 and hMSH2, showing intact nuclear staining in a series of 19 GCTs, suggesting that MMR genes may not play a significant role in early phases and progression of TGCT (*29*). In addition, previous studies showed significantly higher incidence of MSI compared with the unselected series (45 *vs.* 6%) in resistant tumours, both chemo-naïve and pre-treated cases, demonstrating that lacking expression of hMLH1 or MSH6 were significantly more frequent in resistant GCTs (*14*). Velasco *et al.* investigated the expression of the two most mutated MMR genes, MSH2 and MLH1, in sporadic testicular GCTs, founding that 25% of GCTs exhibited increased frequency of MSI. They also investigated MMR gene expression in testicular cancer as a molecular marker for clinical outcome (recurrence, response to chemotherapy and death) using protein expression and specific genetic alterations in 162 patients with testicular GCTs of different histological types (*17*). They found that tumors with altered MMR expression respond differentially to treatment, suggesting that standard platinum-based chemotherapy

does not appear to be very effective in tumors with MMR deficiency as measured by a high frequency of MSI and decreased immunostaining of MSH2 and MLH1(*17*). Al-Obaidy *et al.* analysed a series of TGCT patients including 13 patients with a subsequent contralateral TGCT. Thus, they found MLH1, PMS2, MSH2, and MSH6 retained staining in all cases of bilateral tumors (*30*).

4. Conclusion

Although further investigations on large series will be necessary to obtain more significant data, our results suggest that does not seem to be any substantial differences between adult and pediatric GCTs regarding the expression of MMRs.

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[§]These authors contributed equally to this work.
**Address correspondence to:*
Paolo Chieffi, Department of Precision Medicine, University of Campania "Luigi Vanvitelli", Via L. De Crecchio 80138, Naples, Italy.
E-mail: paolo.chieffi@unicampania.it (Paolo Chieffi)

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