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Influence of hMLH1 Methylation, Mismatch Repair Deficiency and Microsatellite Instability on Chemoresistance of Testicular Germ-cell Tumors

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Abstract. *Background: Cisplatin-based chemotherapy can cure more than 80% of metastatic germ-cell testicular tumors (GCTs). The response to cisplatin-based chemotherapy has been related to Microsatellite Instability (MSI), which is caused by genetic or epigenetic changes in genes of the DNA Mismatch Repair (MMR) pathway. Patients and Methods: We investigated 15 refractory and 36 chemosensitive GCTs for immunohistochemical loss of hMLH1, hMSH2 and hMSH6 protein expressions, in conjunction with hMLH1 gene methylation and MSI of GCTs, with a complete follow-up. Results: A loss of either of the MMR protein expressions was detected in 14 cases (27.5%). Pathological hMLH1 protein expression was seen in 10 cases (19.6%). hMLH1 methylation was found in 11 cases (21.6%) and was highly correlated with loss of hMLH1 expression ($p<0.0001$) and with immuno-histochemically-detected MMR deficiency ($p=0.0005$). MSI was found in 16 cases (31.4%). There was no correlation between hMLH1 methylation and MSI. Neither hMLH1 methylation status, nor MSI correlated with any of the clinicopathological parameters investigated (tumor stage, histology, resistance to systemic treatment). Conclusion: hMLH1 gene methylation was detected as a common alteration in GCTs, and correlated with the loss of hMLH1 protein expression ($p<0.0001$). Neither hMLH1 gene methylation, MMR deficiency, nor MSI showed a relationship with the relevant clinicopathological parameters.*

In industrialized countries, testicular germ-cell tumors (GCTs) account for up to 60% of all malignancies diagnosed in male patients between 20 and 40 years of age (1). GCT is one of the most sensitive tumors to cisplatin-based chemotherapy, since, even in disseminated cases, a remission rate of 80% can be obtained (2). The biological bases of this exceptional chemosensitivity have not been elucidated yet.

It is generally accepted that the cytotoxic activity of cisplatin results from its interactions with DNA (3). DNA-platinum covalent adducts inhibit fundamental cellular processes, including replication, transcription, translation and DNA repair (3). The response to cisplatin-based chemotherapy has been related to Microsatellite Instability (MSI) (4), *i.e.* alterations in the length of the short repetitive sequences of the genome by small deletions or insertions. MSI is caused by genetic or epigenetic changes in genes of the DNA Mismatch Repair (MMR) pathway. Several proteins of this pathway have been identified, including hMLH1, hMSH2 and hMSH6 (5). Methylation of the *hMLH1* gene promoter region has recently been demonstrated to be an important mechanism (approx. 90% of MSI cases) of gene inactivation in cancer (6).

A correlation between MMR deficiency and treatment resistance can be explained by different mechanisms (4): the MSI renders the genome of the cancer cell prone to harbor secondary mutations, which could be responsible for the resistant phenotype. MMR proteins are proved to be directly involved in induction of apoptosis. This could result in resistance to apoptosis, independent of the presence of actual MSI. Nevertheless, loss of MMR functions may prevent the cells from repairing cisplatin-induced DNA damage, thus activating apoptotic pathways.

Although several papers have dealt with MSI in GCT (7-10), the results were controversial. Only Mayer *et al.* (10) found a positive correlation between MSI and treatment resistance in GCTs. Koul *et al.* found *hMLH1* promoter hypermethylation in 6.5% of the tested male GCTs, which also exhibited an absence of or down-regulated gene expression (11). However, we have found no data in the literature regarding the relationship between the methylation of the *hMLH1* gene and MSI in GCTs.

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Table I. *Clinical and pathological characteristics of the patients.*

	No. of cases	
Number of patients	51	(100%)
Mean age	32	(17-60)
5-year survival		91.4%
Median follow-up (months)	52	(2-135)
Histology of the primary tumor		
S	10	(19.6%)
EC	3	(5.9%)
CC	1	(2.0%)
T	6	(11.8%)
S+EC	5	(9.8%)
S+T	2	(3.9%)
S+EC+T	4	(7.8%)
EC+YS	3	(5.9%)
EC+T	11	(21.5%)
EC+YS+T	1	(2.0%)
EC+CC+T	5	(9.8%)
Clinical stage		
Early stage (I,IIA)	23	(45.1%)
Late stage (IIB,IIC,III)	28	(54.9%)
Therapy after semicastration		
Chemotherapy	33	(64.7%)
Chemotherapy+salvage surgery	1	(2.0%)
RLA+chemotherapy	14	(27.4%)
Chemotherapy+radiotherapy	3	(5.9%)
Clinical outcome		
No evidence of disease	5	(9.8%)
Complete response	31	(60.8%)
Partial response	6	(11.8%)
Stable disease	3	(5.9%)
Progressive disease	2	(3.9%)
Died	4	(7.8%)

T, teratoma; S, seminoma; EC, embryonal carcinoma; CC, choriocarcinoma; YS, yolk sac tumor; RLA, retroperitoneal lymphadenectomy.

Our aim was to investigate the MMR protein (hMLH1, hMSH2, hMSH6) expressions, *hMLH1* gene methylation and MSI of GCTs. We correlated each of the above-mentioned gene alterations and/or protein expressions with the clinicopathological parameters of the disease, *i.e.* histology, clinical stage and response to treatment.

Patients and Methods

Patients. Specimens were obtained during the semicastration of 51 patients with testicular tumor, between the years 1993 and 2003, performed at the National Institute of Oncology, Budapest, Hungary. Prior to surgery, the patients received neither radio- nor chemotherapy. The specimens were fixed in 4% buffered formalin for paraffin embedding. Histological examination was performed on hematoxylin- and eosin-stained tissue sections. The tumors were histopathologically classified according to the WHO criteria (12). For clinical staging, physical examination, serum markers of the beta subunit of human chorionic gonadotropin (hCG) and alpha-fetoprotein (AFP) (13), chest X-ray, abdominal ultrasound and computerized tomography (CT) scans were routinely used. Additional CT or magnetic resonance imaging (MRI) was performed if clinically needed. Staging was based on the UICC classification (14). Early stage was defined as stage I or stage II/A. Late stage was defined as stage II/B, II/C or stage III. Therapy was performed according to the institution's protocol (15). The clinical response was measured in accordance with accepted criteria in testis tumor (16). Fifteen patients belonged to the refractory group. Patients were considered refractory when progression or relapse occurred despite adequate initial or salvage treatment. The chemosensitive group was composed of 36 patients. Only patients with a complete remission and relapse-free follow-up for more than 1 year were included. Table I shows the clinical and pathological characteristics of the patients.

Immunohistochemistry. The 4-μm formalin-fixed paraffin-embedded tumor sections were deparaffinized in 2 changes of xylene for 30 minutes each and then hydrated in decreasing concentrations of ethanol and washed in 2 changes of distilled water. Endogenous peroxidase blocking was performed with 3% H_2O_2 for 10 minutes. The slides were washed in 2 changes of distilled water and rinsed in citrate buffer (pH 6). Heat-induced epitope retrieval was performed in a water-bath (97˚C, 35 minutes). After cooling for 5 minutes, the slides were removed to TBS, and primary antibodies (hMLH1: G168-15; BD Biosciences Pharmingen, CA, USA, hMSH2: 25D12; Novocastra, UK, hMSH6: GTBP.P1/66.H6; Serotec Ltd., UK) were applied. The sections were washed 3x5 minutes with TBS, and were incubated with polymer-horseradish peroxidase (EnVision+ System, DakoCytomation, CA, USA) for 30 minutes. The sections were washed twice with TBS, and were incubated with the 3,3'-diaminobenzidine Substrate-Chromogen (DAB) (Dako, CA, USA), slightly counterstained with Mayer's hematoxylin and mounted (17). Paraffin-embedded human tonsil tissues were used as positive controls. The primary antibody was replaced with 3% bovine albumin solution in TBS as a negative control.

DNA isolation. DNA was isolated from paired tumor and normal samples of paraffin-embedded tissues with a High Pure PCR template preparation kit (Roche Diagnostics GmbH, Manheim, Germany), according to the manufacturer's instructions.

Microsatellite instability. Paired tumor and normal template solutions were used in the PCR reactions. Fluorescence-labelled primers were used for the following microsatellite loci: D2S123, D5S346, D17S250 (dinucleotide repeats) and Bat-25, Bat-26 (poly-A repeats) (18). Primer sequences were chosen from the Human Genome Database (http://www.gdb.org). Analysis was performed by the use of an ABI 310 Automated Genetic Analyzer, with GeneScan 3.7 software. Tumor MSI was defined as marker peak shifts compared to the normal samples.

Methylation analysis. Sodium bisulfite conversion of the DNA template was performed, as previously described (19). The methylation analysis

Table II. Primers and probes used for methylation analysis.

	Forward primer	TaqMan probe	Reverse primer
MLH1	**CG**TTATATAT**CG**TT**CG** TAGTATT**CG**TGTTT	6FAM-**CGCGACG**TCAAA**CG** CCACTA**CG**-TAMRA	CTAT**CG**C**CG**CCTCAT**CG**T
ACTB	TGGTGATGGAGGAGG TTTAGTAAGT	6FAM-ACCACCACCCAACACA CAATAACAAACACA-TAMRA	AACCAATAAAACCTACTCCTCCCTTAA

was performed by the fluorescence-based real-time PCR assay, MethyLight, as described previously (20). Primer and probe sets, designed specifically for bisulfite-converted DNA, were used: one set of primer pair and TaqMan probe specific for a fully methylated segment of the *hMLH1* promoter and a reference set for β-actin (ACTB), to normalize for input DNA. The *ACTB* target did not contain any CpGs. Human sperm DNA was used as negative control, and SssI methylase (New England Biolabs)-treated human lymphocyte DNA was used as positive control. The percentage of fully methylated molecules (PMR) at the specific locus was calculated by dividing the *GENE:ACTB* ratio of the sample by the *GENE:ACTB* ratio of the positive control and multiplying by 100. Considering the average value and average scatter of normal samples, a sample was assessed as hypermethylated if the result reached a minimum of 10 PMR. The primers and probes used in the methylation analysis were as described by Eads *et al.* (21) (Table II). The real-time PCR assays were performed by the use of the ABI –7900 Sequence Detection System. The PCR mix contained 0.6 μM of each primer, 0.2 μM probe, 5.5 μM $MgCl_2$ and JumpStart Taq ReadyMix (Sigma, USA) in a final volume of 25 μl. The thermal profile was: 95˚C, 5 minutes and 45 cycles of 95˚C 15 seconds and 60˚C 1 minute.

Statistical analysis. Binomial variables were compared by the two-tailed Fisher's exact probability test. The comparison of 2 unpaired groups was analysed by the Mann-Whitney test. The association between two variables was quantified with Spearman correlation. For survival analysis, curves were calculated using the Kaplan-Meier method and were compared by the log-rank test. A probability ≥95% ($p<0.05$) was considered to present statistical significance. The SPSS 11.0 for Windows® (SPSS Inc., IL, USA) was used for calculations.

Results

A loss of either of the MMR protein expressions was detected in 14 cases (27.5%). In cases with intact intranuclear reactivity, the intensity of staining was higher in tumor cells than in normal stromal lymphocytes (Figure 1A) Pathological hMLH1 protein expression was seen in 10 cases (19.6%) (Figure 1B). In 1 case, all of the MMR protein expressions were pathological. In 4 cases, hMSH6 protein expression was lost. Three cases with loss of hMSH6 also showed loss of hMSH2 expression. We found no isolated loss of hMSH2 expression. A strong intranuclear expression was detected for all MMR-related proteins in intratubular germ cell neoplasia (IGCNU).

We found *hMLH1* hypermethylation in 11 cases (21.6%), of which 3 expressed hMLH1 protein strongly. However, 2 cases with loss of hMLH1 protein expression showed no hypermethylation (Table III). hMLH1 methylation was highly correlated with loss of nuclear hMLH1 expression ($p<0.0001$) and with immunohistochemically-detected MMR deficiency ($p=0.0005$).

In addition, *hMLH1* methylation was not detected in any but 1 case in the refractory group. Four deaths occurred in this series, all of them belonging to the *hMLH1* non-methylated group. However, the survival curves of the *hMLH1* methylated *vs.* non-methylated groups did not differ significantly ($p=0.2354$).

The *hMLH1* methylation status was correlated with tumor stage, histology (seminoma *vs.* non-seminoma), p53 expression, apoptosis index (unpublished results), but no significant relationship was observed with any of these parameters.

MSI was found in 16 cases (31.4%) (Figure 2), however no sample showed high MSI. In 3 cases shift was detected in 2 of the 5 loci analyzed. The proportion of MSI in the refractory group and in the sensitive group was 27.8% and 32.4%, respectively. The MSI status did not correlate with any of the clinicopathological parameters investigated (*hMLH1* methylation, loss of MMR expression, tumor stage, histology, p53 expression, apoptosis index [unpublished results], or resistance to systemic treatment).

Discussion

Since cisplatin (CDDP) is a DNA-damaging agent, the ability of a cell to survive exposure could depend on the efficacy of its DNA-repair pathways. Correlations between activation of MMR mechanisms and responses to CDDP have been reported (22). *In vitro* analyses of cell lines of various origins have suggested a correlation between MMR, MSI and sensitivity toward CDDP (23). Response to CDDP-based chemotherapy of ovarian carcinoma has been related to MSI (5).

A large majority of MSI is caused by the *hMLH1* methylation in sporadic colorectal cancer (24), gastric carcinoma (25) and endometrial carcinoma (26), all of

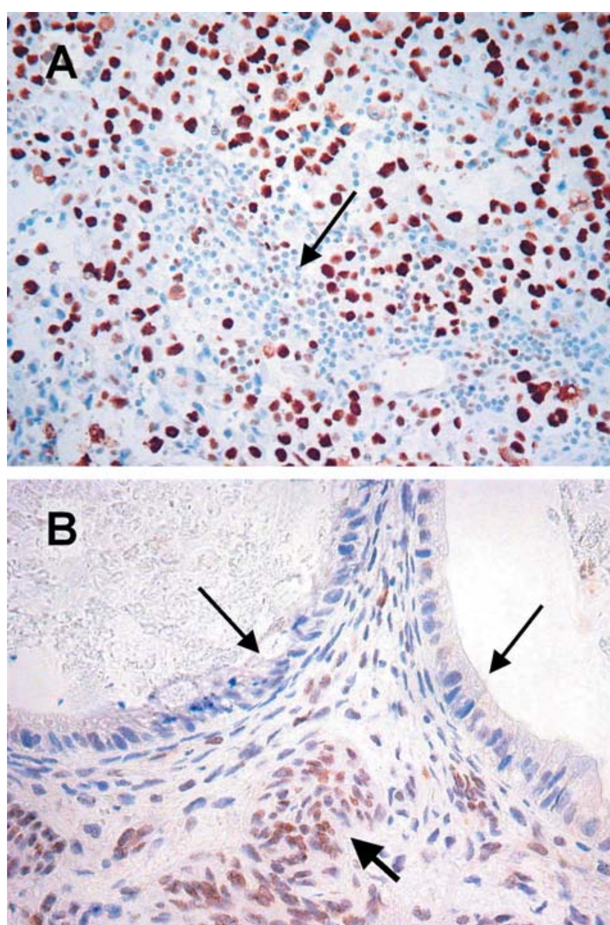

Figure 1. *A, Seminoma. Strong intranuclear staining for hMSH2. The reaction is more intense in tumor cells than in the tumor infiltrating normal lymphocytes (arrow) (IHx100); B, Mature teratoma. Loss of hMLH1 protein in the epithelial component (thin arrows). Normal hMLH1 expression in the stromal component (thick arrow) (IHx100).*

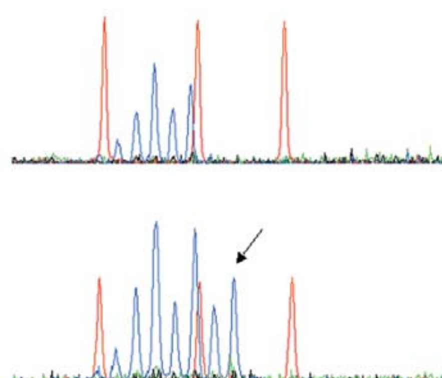

Figure 2. *Microsatellite instability phenotype. The arrow indicates a new allele of the D17s250 marker appearing in the tumor sample.*

which are known to have a high frequency of MSI. No previous investigations have been performed, however, on the role of *hMLH1* methylation in the MSI of GCTs.

Koul *et al.* found *hMLH1* hypermethylation in 6.5% of GCT cases (11). They showed that methylation of the promoter region between –269 and –196 from the transcriptional start was associated with absent or down-regulated hMLH1 expression. We found *hMLH1* methylation in 21.6% of cases in the distal promoter region between –662 and –575. *hMLH1* methylation correlated well with loss of hMLH1 expression ($p<0.0001$) and with MMR deficiency (loss of either of MMR proteins) ($p=0.0005$). However, we detected 2 cases with loss of hMLH1 protein expression that showed no *hMLH1* methylation. This suggests that hMLH1 deficiency can be caused by mechanisms other than hypermethylation of the *hMLH1* gene, (*e.g.* allelic loss, mutations). In 3 cases, strong hMLH1 protein expression was found in parallel with *hMLH1* methylation. We did not systematically record heterogeneity in the staining pattern; however, one possible explanation for this finding could be that the RT-PCR assay is likely to be more sensitive in detecting small clones of tumors with *hMLH1* methylation than immunohistochemistry.

Mayer *et al.* (10) found no correlation between MSI and immunohistochemical assessment of hMLH1, hMSH2 and hMSH6. We also found no relationship between *hMLH1* methylation and the MSI of tumor samples. Seventy-five percent of the MSI tumors did not show *hMLH1* methylation. On the other hand, no MSI was found in 7 *hMLH1*-methylated cases. Since *hMLH1* methylation correlated well with loss of MMR protein expressions, one reasonable explanation for this discordance is that MSI is caused – at least partly – by other MMR factors, such as PMS1 and PMS2, as has been suggested for prostate cancer (27).

MMR deficiency leads to further mutations that may contribute to the oncogenic process, which could result in a more aggressive phenotype. No correlation was found, however, with stage of tumor, or resistance to systemic treatment. Moreover, a trend toward survival benefit was observed in the *hMLH1*-methylated group in comparison with samples containing non-methylated *hMLH1*, since *hMLH1* methylation was not detected in any but 1 refractory case. Furthermore, all patients with *hMLH1*-methylated tumors are alive, while 4 deaths occurred in the *hMLH1*-non-methylated group. The difference of Kaplan-Meier survival curves, however, was not significant, mainly because of the low number of cases. Koul *et al.* recently described *de novo* promoter hypermethylation of several genes (*RASSF1A*, *HIC1*, *APC*, *BRCA1*, *MGMT*, *RARB*) following cisplatin treatment (28). In the case of *BRCA1*,

Table III. *Correlation of hMLH1 protein expression and hMLH1 methylation in 51 testicular germ cell tumors.*

hMLH1 gene methylation	hMLH1 protein expression		Total	*p*
	Weak, absent	Strong		
Positive	8	3	11	<0.0001
Negative	2	38	40	
Total	10	41	51	

MGMT and *RARB,* cisplatin-sensitive tumors had the highest incidence of promoter hypermethylation, but this was decreased or absent in highly-resistant tumors. While the other 3 genes showed the highest hypermethylation rate in the highly-resistant tumors. Our results seem to support that *hMLH1* belongs to the previous group of hypermethylation, which favors the treatment response. The survival benefit of *hMLH1*-methylated cases could be explained by the decreased DNA repair ability after CDDP therapy. Without additional evidence, however, these considerations remain speculative at this point.

The positive predictive value of *hMLH1* methylation and MSI is well documented in sporadic colorectal cancers, where MSI shows a relationship with diploid status and absence of p53 protein expression. However, most of the GCTs are aneuploid, and the *p53* mutation rate is generally low (about 5-7%). We found no correlation between *hMLH1* methylation and p53 expression (data not shown), or any other relevant clinicopathological parameter. Loss of hMSH6 protein expression occurred in 4 cases, while in 3 of them, loss of hMSH2 expression was observed as well. Separate loss of hMSH2 expression was not observed. This is in line with the report by Guerrette *et al.* (29), who suggested that hMSH2 and hMSH6 exist as heterodimeric proteins, and by Acharya *et al.* (30), who demonstrated a protein-protein cross-linking between hMSH2 and hMSH6.

No MMR protein loss was detected in the tumor cells of IGCNU. Moreover, the staining in these cells was stronger than in the surrounding normal cells. This suggests that MMR deficiency is not an early defect and has no role in *in situ* tumorigenesis.

Several groups have analyzed MSI and the MMR pathway in GCT and found no correlation between the results and clinical outcome (7-9). The only exception is the paper by Mayer *et al.* (10), who reported a positive correlation between MSI and treatment resistance in GCT. In their work, the resistant tumors showed a significantly higher incidence of MSI compared with the unselected series ($p<0.001$). We found no relationship between MSI and the clinical outcome of tumors. Moreover, MSI was not correlated with tumor stage, histological type, *hMLH1* methylation, MMR status, p53 expression, or apoptosis index, although no sample showed high MSI status.

Our results suggest that, in contrast to familiar colorectal cancer and several other solid tumors (24), *hMLH1* methylation and MMR (hMLH1, hMSH2, hMSH6) protein deficiency do not contribute significantly to the MSI status of GCTs. Moreover, MSI does not correlate with either the most important pathological parameters, or with the clinical outcome of GCTs.

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