Colorectal cancer screening for average- and high-risk individuals: beyond one-size-fits-all

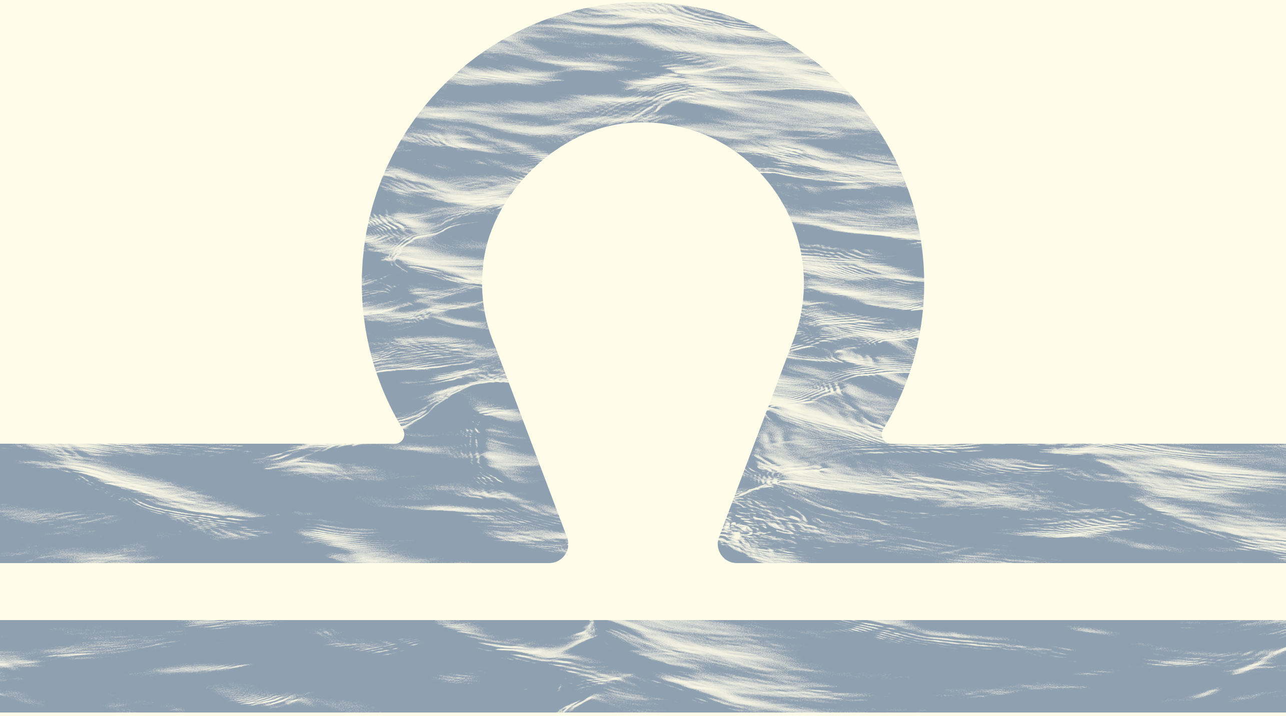
Breekveldt, E.C.H.

Citation

Breekveldt, E. C. H. (2024, June 5). *Colorectal cancer screening for average- and high-risk individuals: beyond one-size-fits-all*. Retrieved from https://hdl.handle.net/1887/3759760

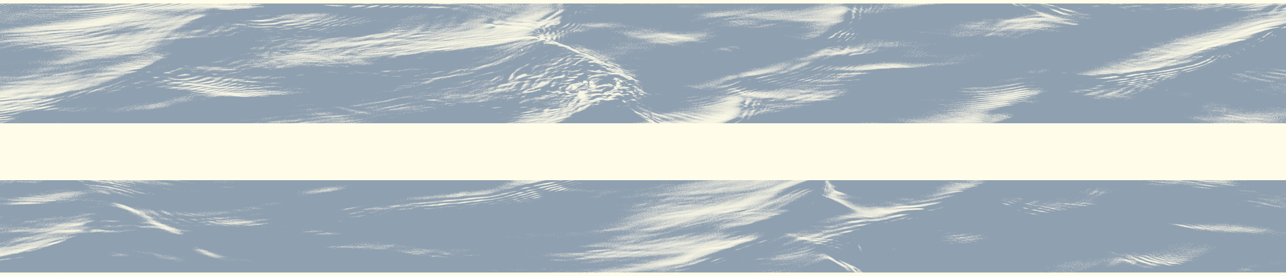
Part III

Colorectal cancer in testicular cancer survivors treated with platinum-based chemotherapy



Chapter 9

Somatic hits in mismatch repair genes in colorectal cancer among non-seminoma testicular cancer survivors



BLM Ykema, ECH Breekveldt, B Carvalho, T van Wezel, GA Meijer, M Kerst,
M Schaapveld, FE van Leeuwen, P Snaebjornsson, and ME van Leerdam.

BRITISH JOURNAL OF CANCER 2022

ABSTRACT

Background

Non-seminoma testicular cancer survivors (TCS) have an increased risk of developing colorectal cancer (CRC) when they have been treated with platinum-based chemotherapy. Previously we demonstrated that among Hodgkin lymphoma survivors (HLS) there is enrichment of rare mismatch repair (MMR) deficient (MMRd) CRCs with somatic hits in MMR genes. We speculate that this phenomenon could also occur among other cancer survivors. We therefore aim to determine the MMR status and its underlying mechanism in CRC among TCS (TCS-CRC).

Methods

Thirty TCS-CRC, identified through the Dutch pathology registry, were analysed for MMR proteins by immunohistochemistry. Next-generation sequencing was performed in MMRd CRCs without MLH1 promoter hypermethylation (n=4). Data were compared with a male cohort with primary CRC (P-CRC, n=629).

Results

MMRd was found in 17% of TCS-CRCs vs.. 9% in P-CRC (p=0.13). MMRd was more often caused by somatic double or single hit in MMR genes by mutation or loss of heterozygosity in TCS-CRCs (3/30 (10%) vs.. 11/629 (2%) in P-CRCs (p<0.01)).

Conclusions

MMRd CRCs with somatic double or single hit are more frequent in this small cohort of TCS compared with P-CRC. Exposure to anticancer treatments appears to be associated with the development of these rare MMRd CRC among cancer survivors.

BACKGROUND

Testicular cancer (TC) survivors have an increased risk of developing colorectal cancer (CRC) (1–7). This increased risk appears to be associated with platinum-based chemotherapy, which was associated with a hazard ratio (HR) for CRC of 3.9 (95% confidence interval (CI) 1.7–8.9) (8,9). Such an association between platinum-based treatment and risk of second primary gastrointestinal (GI) malignancies has also been described in childhood cancer survivors (10).

The increased risk of second primary CRC in TC survivors (TCS-CRC) may be due to mutagenic and genome destabilising effects of cancer treatment on normal colonic mucosa (11). These changes can result in premature ageing of the colonic mucosa and/or cancer development at an earlier age among cancer survivors (12,13). These treatment-induced changes may also activate pathogenetic processes that result in molecular profiles that are different from those of primary CRC. Previously, we have shown that Hodgkin lymphoma (HL) survivors treated with abdominal radiotherapy and/or procarbazine-containing chemotherapy have a higher frequency of mismatch repair (MMR) deficient (MMRd) CRC compared with CRC patients in the general population (14). This higher frequency was due to the enrichment of somatic double hit in MMR genes by either mutations or loss of heterozygosity (LOH). Also, MMRd cases with somatic single hit occurred in this group. These findings suggested a novel association of prior anticancer therapy with somatic MMR gene mutations or LOH. We hypothesise that this association may not be specific to the context of HL. Instead, we contemplate that this phenomenon could also occur in other cancer survivors that received other types of anticancer treatments. To examine this hypothesis, we evaluated whether MMR status and the underlying mechanism of MMRd in TCS-CRC differs from CRC occurring in the general population (primary CRC, P-CRC).

METHODS

Patients and tissue samples

The population-based Netherlands Cancer Registry (NCR) was used to identify CRC after non-seminoma TC, diagnosed before the age of 50 years, irrespective of non-seminoma treatment. Patients were diagnosed with non-seminoma TC between 1989 and 2011. This range is caused by the fact that CRC develops predominately 10 years after treatment for TC, and therefore CRC was still diagnosed in 2019. A total of 36 CRC were identified at least one year after the diagnosis of non-seminoma TC. These cases were subsequently linked to the PALGA (the nationwide network and registry of histopathology and cytopathology) registry to obtain pathology reports and formalin-fixed paraffin-embedded (FFPE) material (15). Tissue from 30 TCS-CRCs was available for analyses. Non-seminoma TC treatment data were retrieved through the NCR. All data collection and analyses were pseudonymised.

Histopathology

Histopathology of 30 of 36 (83%) retrieved samples was reassessed on haematoxylin & eosin (H&E)-stained slides according to standard protocol by an experienced gastrointestinal pathologist (PS). One patient had a metachronous CRC, of which both CRCs were completely evaluated, leading to 30 CRCs in 29 TC patients.

Immunohistochemistry

Immunohistochemistry (IHC) was performed for MMR proteins according to standard protocols for Ventana immunostainer (MLH1 (Agilent/DAKO, Cat. # M3640), MSH2 (Roche/Ventana, Cat. # 8033684001), MSH6 (Epitomics, cat. # AC-0047EU), PMS2 (Roche/Ventana, Cat. # 8033692001). IHC was performed on tissue microassay when available. In case of biopsy material, whole sections were cut for IHC.

Molecular analyses

The AllPrep DNA/RNA FFPE extraction kit (QIAGEN, Germany) was used to isolate DNA of FFPE material of CRC in TC survivors following the manufacturer's instructions. The concentrations were measured using the Qubit 2.0 Fluorometer with the Qubit dsDNA Assay Kit (Provenience).

Additionally, we evaluated the mutational status in common CRC-related genes, i.e. KRAS, NRAS, BRAF and PIK3CA, using a gene panel (Sequenom Massarray, Agena Bioscience, San Diego, California, USA) that also included AKT1, DDR2, EGFR and MEK1.

Due to very high concordance of MMR IHC and MSI PCR between between MMR status and microsatellite status in colorectal cancer (16–19), we did not perform MSI PCR.

Assessment of mechanism behind MMR deficiency

Promoter methylation of MMR genes was evaluated in MMRd tumours by a multiplex ligation-dependent probe amplification (MLPA) kit (ME011-B2 kit; MRC Holland, Amsterdam, the Netherlands). This probemix included a total of 25 probes for the promoter region of six different MMR genes (MLH1, MSH2, MSH6, PMS2, MSH3, MLH3). Gene positivity was defined as 33% of probes per gene with a cut-off for positivity of 0.2 at probe level.

In case of MMRd without MLH1 promoter methylation, further analysis was performed on both tumour tissue and normal tissue to screen MMR genes for mutations and LOH via Next Generation Sequencing (NGS) using the msCRCv2 panel with supplier's materials and protocols (Life Technologies, Carlsbad, CA, USA) as described previously (20). Details of the panel can be found at https://www.palga.nl/datasheet/LUMC/MMR_Panel_MSCRCv2_LUMC.pdf.

The mechanism underlying MMRd was classified as follows: (1) MLH1 promoter methylation, (2) Lynch syndrome, (3) somatic double hit by mutations or LOH and (4) somatic single hit by mutation or LOH. For statistical analysis, cases with somatic double or single hit were grouped together. We included all cases of MMRd in our analysis, including MMRd explained by Lynch syndrome to provide an overview on all MMRd subgroups.

Control group of CRC <70 years in the general population

The frequencies of MMRd and its mechanism of inactivation were compared to data of sporadic CRC in a general population cohort, referred to as primary CRC (P-CRC) (21,22). This included 1,117 patients prospectively collected between 2007 and 2009 at ages ≤70. For this study, we selected male patients (n=629) only to ensure comparability with our cohort. This control group was selected because it was a relatively young cohort within the general population and because of the availability of the required data (MSI status, MMR status, MLH1 promoter methylation, etc).

Statistical analyses

Data was analysed using IBM SPSS V.22.0 database software. Data were compared between groups using $\chi 2$ tests or Fisher's exact tests for categorical data and Mann–Whitney U-test for continuous data that were not normally distributed. The significance level was defined as two-sided $p \leq 0.05$.

RESULTS

Patient characteristics

FFPE material of 30 out of 36 TCS-CRCs (83%) was available for analyses (Figure 1). One TC survivor had developed a second CRC after 1 year. The non-seminoma TC were diagnosed at a median age of 39 years (IQR 22–45 years) in the 29 patients (Table 1). In most cases, data on TC therapy could not be retrieved. Of patients for whom data could be retrieved (n = 9), all had received platinum-based chemotherapy (8/9 cisplatin and 1/9 carboplatin). Patient characteristics of the non-seminoma TC are described in Table 1.

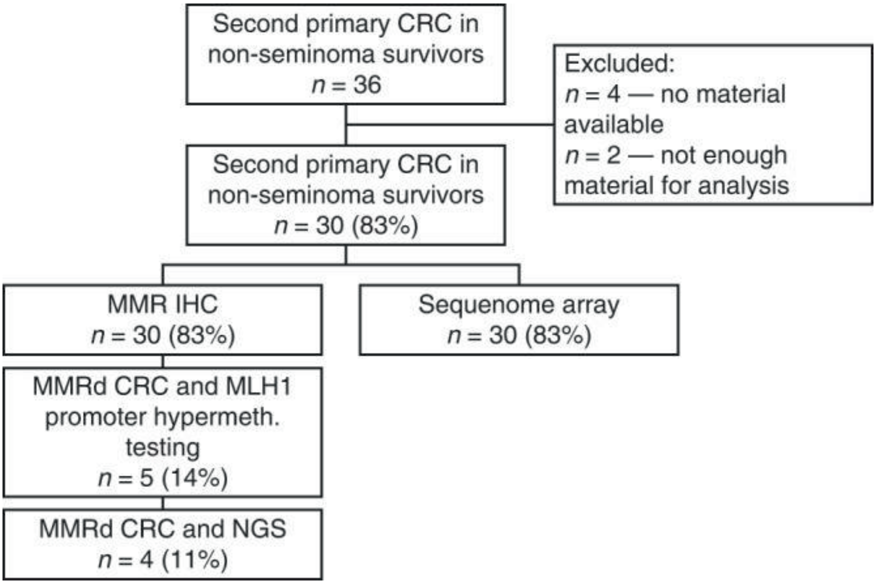


Figure 1 – Study flowchart. The flowchart of colorectal cancer (CRC) diagnosed in non-seminoma testicular cancer survivors treated with platinum-based chemotherapy.

Table 1 - Baseline characteristics of non-seminoma testicular cancer (TC) survivors with second primary colorectal cancer (CRC).

	N (%) (N = 29)*
Age of non-seminoma TC diagnoses	
Median (range)	39 (22–45)
Treatment period	
1989–1999	22 (76%)
2000–2011	7 (24%)
Stage non-seminoma	
I	9 (40%)
II	3 (15%)
III	4 (20%)
IV	4 (20%)
Unknown	9
Treatment non-seminoma	
Chemotherapy only	8 (89%)
Radiotherapy + chemotherapy	1 (11%)
Unknown	20

*Only characteristics of those patients from whom samples were retrieved are presented in the table of which one patient developed two CRCs.

The median interval between non-seminoma TC diagnosis and CRC was 19 years (IQR 2–29 years). Median age at diagnosis of TCS-CRC was 55 years (range 35–68), which was significantly younger than the median age at diagnosis of the P-CRC (diagnosed ≤70 years) (61 years, IQR 27–71 years, $p<0.01$). The tumour location did not significantly differ between TCS-CRC and P-CRC. All TCS-CRC (n = 30) were conventional adenocarcinomas. KRAS, NRAS and BRAF mutation occurred in 35, 7 and 3% of TCS-CRCs, respectively. Patient and CRC characteristics are described in Table 2.

Table 2 - Characteristics of second primary colorectal cancer (CRC) in non-seminoma survivors and primary CRC.

	Second primary CRC in non-seminoma survivors (n=29)	*Primary CRC <70 years (n=629)*	*P value*
Interval between TC diagnosis and CRC (median, range, years)	19 (2–29)	N/A	–
Age at diagnosis of CRC (median, range, years)	55 (35–68)	61 (27–71)	<0.01
Year of CRC diagnosis (range)	1994–2019	2007–2009	N/A
	Total CRC n = 30 (n, (%))	*Total CRC n = 629 (n, (%))*	
Location			0.59
Proximal*	8 (29%)	153 (25%)	
Distal	12 (43%)	218 (36%)	
Rectum	8 (28%)	228 (38%)	
Unknown	1	30	
Stage			0.18
I	10 (50%)	123 (28%)	
II	3 (15%)	123 (28%)	
III	6 (30%)	173 (39%)	
IV	1 (5%)	25 (6%)	
Unknown	9	184	
MMR status			0.13
Proficient	25 (83%)	575 (91%)	
Deficient	5 (17%)	54 (9%)	
MMR staining			0.20
Staining present	25 (83%)	576 (92%)**	
MLH1 and PMS2 deficiency	3 (10%)*	38 (6%)	0.38
MSH2 and/or MSH6 deficiency	2 (7%)	14 (2%)	0.12
Mechanism of MMR deficiency			0.02
Somatic *MLH1* hypermethylation	1 (3%)	30 (5%)	0.18
Lynch syndrome	1 (3%)	13 (2%)	0.64
Somatic double or single hit in MMR genes	3 (10%)	11 (2%)	<0.01

*In one there was loss of MLH1 and PSM2 staining, which also included secondary loss of MSH6 staining.

**One case with MMR proficient IHC result while MSI PCR showed MSI.

MMR status of second primary colorectal cancer in non-seminoma survivors

MMRd occurred in 17% (5/30) of TCS-CRC compared with 9% (54/629) in P-CRC (p=0.13). Three of five MMRd cases (60%) demonstrated combined absence of MLH1 and PMS2 staining. One of these cases also showed absence of MSH6 staining, which is recognised as secondary inactivation resulting in loss of MSH6 on IHC (23). The remaining two cases demonstrated either isolated absence of MSH6 staining or combined absence of MSH2 and MSH6 staining. Of all five MMRd cases, treatment given for non-seminoma TC was unknown.

Underlying mechanism of MMR deficiency in colorectal cancer in non-seminoma survivors

Of the three cases with MLH1/PMS2 deficiency, the first one had somatic hypermethylation of the MLH1 promoter. The second was explained by Lynch syndrome (germline MLH1 mutation accompanied by second somatic hit) and the third case by somatic double hit in the MLH1 gene by mutation and LOH (Table 3). In the fourth case, which demonstrated MSH2/MSH6 deficiency on IHC, there was somatic single hit in the MSH2 gene by LOH. In this case, we also detected LOH of MSH6, but these genes are in close proximity of each other on chromosome 2. It was therefore classified as a somatic single hit. Finally, for the case with isolated MSH6 deficiency, we found three mutations in the MSH6 gene (Table 3). These three mutations included one frameshift mutation with known pathogenicity and two missense mutations of unknown pathogenicity. Therefore, we classified this case as having somatic single hit.

Table 3 - Outcome of next-generation sequencing (NGS) of the four mismatch repair deficient (MMRd) colorectal cancer (CRC) in non-seminoma testicular cancer (TC) survivors (exclusion of the MMRd CRC explained by MLH1 hypermethylation).

No.	Age at TC	Age at CRC	IHC loss	Material	Chr:ChrPos	Gene	HGVS. Coding	Class**	Type	LOH	Conclusion of MMR	Mechanism of MMR deficiency
1*	37	40	MLH1 PMS2	Tumour	3:37053310 3:37038205	MLH1 MLH1	NM_000249.3:c.546-1G>A NM_000249.3:c.207+5G>A	4 4	Splice-site Splice-effect	No LOH	2 mutations	Lynch syndrome
				Normal	3:37053310	MLH1	NM_000249.3:c.546-1G>A	4	Splice-site	No LOH	1 mutation	
2	31	57	MLH1 PMS2	Tumour	3:37053595	MLH1	NM_000249.3:c.677+5G>T	4	Splice-site	LOH of *MLH1*	1 mutation + LOH	Somatic double hit
				Normal		No pathogenic mutation						
3	22	50	MSH2 MSH6	Tumour		No pathogenic mutation				LOH of *MSH2* and *MSH6*	LOH	Somatic single hit
				Normal		No pathogenic mutation						
4	26	35	MSH6	Tumour	2:48026606	MSH6	NM_000179.2:c.1484delG NM_000179.2:c.890C>A NM_000179.2:c.728G>A	4 3 3	Frameshift Missense Missense	No LOH	1 mutation	Somatic single hit
				Normal		No pathogenic mutation						

Abbreviations: TC: testicular cancer, CRC: colorectal cancer, IHC: immunohistochemistry of mismatch repair (MMR) genes, Chr:ChrPos chromosome and chromosome position, HGVS.: a series of variance on one chromosome, LOH: loss of heterozygosity.

* Of one CRC, two samples of FFPE material were available.

** Class: prediction of pathogenicity of gene variant (benign (1), likely benign (2), uncertain (3), likely pathogenic (4) or definitely pathogenic (5)).

The distribution of molecular mechanisms underlying the MMRd was different between TCS-CRC and P-CRC (p=0.02; Table 2). This difference was primarily due to enrichment of MMRd cases showing somatic double or single hit in MMR genes by mutation/LOH (10 vs.. 2%, p<0.01). The frequency of MLH1 promoter hypermethylation was similar to the P-CRC cohort (resp. 3 vs.. 5%, p=0.18). Also, the frequency of Lynch syndrome was similar in TCS-CRC compared with P-CRC (resp. 3 vs.. 2%, p=0.48).

DISCUSSION

In this study, we aimed to determine whether TCS-CRC have different pathogenesis compared to P-CRC for which we evaluated the MMR status and its underlying mechanism. We have found that 17% of TCS-CRC are MMRd. MMRd status is significantly more often caused by double or single somatic hit compared to P-CRC (10 vs. 2%, $p<0.01$). In other words, we have shown that a rare subgroup of CRC with MMR deficiency, i.e. CRC with somatic double or single hit in MMR genes by mutation or LOH, is more common in TCS-CRC. Cases explained by MLH1 promoter hypermethylation or Lynch syndrome are equally frequent in both cohorts.

In a previous study on HL survivors, we demonstrated a significant enrichment of somatic double hit as cause of MMRd (7/54, 13%) compared to the general population (8/1,111, 0.7%) (14). In that study, we primarily focussed on cases demonstrating somatic double hit, but we also found significantly more cases with somatic single hit (3/54, 6%) compared to CRC in the general population (3/1,111, 0.3%, $p<0.01$). The combined frequency of these two rare MMRd subgroups was 19% (10/54), which is much higher than in the general population reference cohort for that study (11/1,111, 1%, $p<0.01$).

The present data show an enrichment of a rare subgroup of MMRd cases, i.e. with somatic double or single hit in MMR genes, as previously observed in the study on HL survivors (14). This enrichment becomes more apparent when comparing these frequencies to data from a recent meta-analysis taking all age-groups into account which showed that somatic double and single hit in MMR genes only occurs in 1.8% and 0.7% of all CRCs, respectively (24). This underscores the rarity of this MMR subgroup in CRC in the general population and contrasts the frequency among second primary CRC. These data are of great importance, because the repeated link between anticancer treatment and the occurrence of these rare MMRd CRC among cancer survivors raises the question whether various anticancer treatments may cause the development of this MMRd subgroup among cancer survivors. The patient cohort with HL survivors was predominately treated with alkylating agents such as procarbazine and/or radiotherapy, while the large majority of patients with non-seminoma TC are treated with platinum-based chemotherapy (25). In the current study, we unfortunately did not have information on treatment of patients with MMRd CRC. Also, experimental data explaining the mechanisms underlying these associations is lacking. Still, there is a link between the MMR system and cisplatin exposure, as it was shown that the MMR mechanism is important in repairing DNA damage caused by cisplatin (26–30). Furthermore, a link between the MMR system, radiotherapy and alkylating agents has been described (14). We previously hypothesised that pre-existing epithelial intestinal cells with some level of MMR dysfunction are targeted by anticancer treatments, which could then lead to the development of MMRd CRC.

Previously, patients with MMRd CRC have been referred to as having Lynch-like syndrome (LLS) when neither MLH1 promoter hypermethylation nor germline mutations in MMR genes were detected. Since then, it has become clear that in a significant part of these cases, acquired somatic double or single hit in MMR genes can be found (31). Cases with double hit in MMR genes can be regarded as fully clarified. However, MMR deficient cases with only a single detectable hit in an MMR gene are not fully clarified. Since inactivation of both alleles is necessary to result in complete loss of expression of MMR genes it can be deduced that a second hit is present although it was not identified. The lack of second hit is most likely explained by genetic alterations that are not detected by the methods used, such as certain types of LOH, epigenetic alterations or complex genomic alterations resulting in silencing of the other MMR gene. In studies examining patients with LLS, there also remains a subgroup where no somatic changes can be detected (31).

In our analysis, we found one TC survivor with corresponding MMR gene mutation both in CRC tumour tissue as well as in normal colonic tissue. Therefore, this single patient was regarded to have Lynch syndrome. The remaining patients did not carry MMR mutations in normal colonic tissue. For these patients it could therefore be concluded that the MMR gene hits were unique to the CRC and not involved in the carcinogenesis of the prior testicular cancer. An increased risk of testicular cancer among Lynch syndrome patients has never been reported (32) and 97% of germ cell tumours from various locations among Lynch syndrome patients are microsatellite stable (33). Also, the rate of MMRd in testicular cancers has been reported to be very low, i.e. much less than 1% (34,35). These observations contrast the relatively high percentage of MMRd in second primary CRC among TC survivors and agree with our finding that second primary MMRd CRC of TC survivors are largely unrelated to Lynch syndrome. This is also analogous to our previous findings on second primary MMRd CRC among Hodgkin lymphoma survivors (14).

Limitations of this study are the small sample size and the incomplete information on prior treatment for non-seminoma TC. Studies on MMRd CRC with somatic double or single hit usually lack information on whether these patients received previous anticancer therapy (31,36–38). However, when combining results from three recent studies with a total of 30 patients with MMRd due to somatic double hit, one of these patients had a previous history of HL and another of leukaemia (39–41). None of these studies reported other prior cancer types or anticancer therapies. Even though treatment for TC was unknown in most cases in the present study, a large majority of non-seminoma TC patients do receive treatment with platinum-based chemotherapy, as the relapse risk varies between 15 and 50% depending on the presence of lymph-vascular invasion (25). Clinical experience shows that a majority of the patients treated for TC will have received chemotherapy and, to a lesser extent, radiotherapy. The increased risk for developing CRC appears to be associated with the dosage of platinum-based chemotherapy in TC survivors (1–10,42). An elevated risk of developing CRC was even present 35 years after treatment (4,5,42). We suggest that platinum-containing chemotherapy is associated with this increased risk, especially since platinum levels in serum remain elevated for a long period after treatment and is still detectable in tissues of various organs (43–47). However, whether long-term retention in colorectal tissue, a fast-turnover tissue, is possible, remains unknown.

To conclude, somatic double or single hit in MMR genes is significantly more frequent in secondary CRCs that develop in non-seminoma TC survivors compared to primary CRC in the general population. Since similar results were shown in HL survivors, this may suggest an association between prior anticancer treatment and MMRd with double or single hit in MMR genes.

Furthermore, our results could imply that this phenomenon is neither specific to a certain primary cancer nor a single type of prior anticancer treatment. These findings need confirmation in larger cancer survivor cohorts.

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