Published in final edited form as:

J Med Genet. 2008 March ; 45(3): 142–146. doi:10.1136/jmg.2007.053850.

Polymorphic *MLH1* and risk of cancer after methylating chemotherapy for Hodgkin lymphoma

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Abstract

Background and objective—Methylating agents are effective chemotherapy agents for Hodgkin lymphoma, but are associated with the development of second primary cancers. Cytotoxicity of methylating agents is mediated primarily by the DNA mismatch repair (MMR) system. Loss of *MLH1*, a major component of DNA MMR, results in tolerance to the cytotoxic effects of methylating agents and persistence of mutagenised cells at high risk of malignant transformation. We hypothesised that a common substitution in the basal promoter of *MLH1* (position –93, rs1800734) modifies the risk of cancer after methylating chemotherapy.

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Author contributions: LJW designed and performed research. AGS designed research and analysed data. KS performed research. LBT was principal investigator of the Hodgkin lymphoma-breast cancer study. JMA developed the hypothesis, coordinated the study, designed research and performed research. All other authors are listed alphabetically, each making a significant contribution to one of the individual components of the study (leukaemia or breast cancer). GJM contributed to the identification and collection of therapy related leukaemia cases.

Competing interests: None.

Ethics approval: Ethical committee approval was obtained for this study.

Patient consent: Informed consent was provided according to the Declaration of Helsinki

Methods—133 patients who developed cancer following chemotherapy and/or radiotherapy (n=133), 420 patients diagnosed with de novo myeloid leukaemia, 242 patients diagnosed with primary Hodgkin lymphoma, and 1177 healthy controls were genotyped for the *MLH1* –93 polymorphism by allelic discrimination polymerase chain reaction (PCR) and restriction fragment length polymorphism assay. Odds ratios and 95% confidence intervals for cancer risk by *MLH1* –93 polymorphism status, and stratified by previous exposure to methylating chemotherapy, were calculated using unconditional logistic regression.

Results—Carrier frequency of the *MLH1* –93 variant was higher in patients who developed therapy related acute myeloid leukaemia (t-AML) (75.0%, n=12) or breast cancer (53.3%. n=15) after methylating chemotherapy for Hodgkin lymphoma compared to patients without previous methylating exposure (t-AML, 30.4%, n=69; breast cancer patients, 27.2%, n=22). The *MLH1* –93 variant allele was also over-represented in t-AML cases when compared to de novo AML cases (36.9%, n=420) and healthy controls (36.3%, n=952), and was associated with a significantly increased risk of developing t-AML (odds ratio 5.31, 95% confidence interval 1.40 to 20.15), but only in patients previously treated with a methylating agent.

Conclusions—These data support the hypothesis that the common polymorphism at position –93 in the core promoter of *MLH1* defines a risk allele for the development of cancer after methylating chemotherapy for Hodgkin lymphoma. However, replication of this finding in larger studies is suggested.

The development of a second primary cancer is a serious late complication of treatment for primary cancer, including Hodgkin lymphoma.¹² Methylating chemotherapy agents, such as procarbazine, are risk factors for the development of second primary cancers, particularly treatment related acute myeloid leukaemia (t-AML).³ More recently, a strong dose–response relation (p trend <0.001) has been demonstrated for procarbazine and the development of second primary lung cancer,⁴ and concern exists that chemotherapy for Hodgkin lymphoma may be associated with the development of other solid tumours.⁵

Methylating agents can modify several atoms in DNA, but methylation of the O⁶ atom of guanine is considered to be of most biological significance since it is both cytotoxic and mutagenic.⁶ Cytotoxicity of O⁶-methylguanine is mediated primarily by the DNA mismatch repair (MMR) pathway.⁷ The actions of MLH1, MSH2 and other proteins correct DNA mismatches that spontaneously occur during replication.⁸ However, replication across a template O⁶-methylguanine can also generate a base-base mismatch that is recognised by DNA MMR,⁹ but rather than repairing O⁶-methylguanine-containing mismatches, MMR initiates cell death by a mechanism which remains to be fully elucidated.¹⁰ As such, loss of MLH1 or MSH2 function renders cells tolerant to the killing effects, but sensitive to the mutagenic effects of methylating agents.⁷

The risk of developing a second primary cancer following treatment for a first cancer can be affected by constitutional genetic polymorphisms that modify cellular drug response pathways.¹¹ Given the importance of DNA MMR in mediating cellular response to methylating agents, we hypothesised that a common G>A polymorphism at position –93 (rs1800734) in the core promoter of *MLH1* modifies risk of developing t-AML and breast cancer after methylating chemotherapy. The *MLH1* –93 G>A polymorphism has previously

been associated with risk of primary lung,¹² colorectal¹³ and endometrial¹⁴ cancer, and is predicted to confer a functional effect on protein expression.¹⁵

PATIENTS AND METHODS

Study populations

Four groups were investigated: population 1 comprised 96 UK patients with pathologically confirmed AML that developed 2 or more months after chemotherapy and/or radiotherapy (table 1). The type of antecedent malignancy was known for 55 patients, and included 15 with breast cancer and 13 with Hodgkin lymphoma. Agent specific data on previous chemotherapy and radiotherapy were available for 81 (84%) cases, including 40 who had radiotherapy alone and 56 who had some form of chemotherapy either alone or in addition to radiotherapy. Twelve of the 96 patients had received a chemotherapeutic methylating agent (procarbazine or dacarbazine), and all of the 12 had been treated for a first primary Hodgkin lymphoma. Populations 2a and 2b comprised 420 patients with pathologically confirmed de novo AML and 952 matched controls (without leukaemia), respectively, who participated in a previous study of acute leukaemia conducted in the UK (table 1).¹⁶ Population 3 comprised 37 women treated for Hodgkin lymphoma at age 30 years or younger in North America and Scandinavia who subsequently developed pathologically confirmed breast cancer one or more years after treatment (table 1).¹⁷ Agent-specific data on previous therapy were available for all patients, with 15 having received treatment that included methylating agents. Populations 4a and 4b comprised 242 primary Hodgkin lymphoma cases and 225 matched controls, respectively, recruited as part of a lymphoma case-control study conducted in the UK.¹⁸

Ethical committee approval was obtained for this study. Informed consent was provided according to the Declaration of Helsinki and the study was approved by the University of York Institutional ethical review committee (Department of Biology).

MLH1 –93 polymorphism analysis

MLH1 –93 status was determined using either allelic discrimination PCR (forward primer 5' CATTCAAGCTGTCCAATCAATAGCT3', reverse primer 5' CGTCTAGATGCTCAACGGAAGTG3', G probe 5' fam-TCACGTTCTTCCTTCAGCTTACG3', A probe 5' tet-CTCACGTTCTTCCTTTAGCTGTAGCTTACGC3') or polymerase chain reaction restriction fragment length polymorphism assay.¹⁹ Assay accuracy was determined by direct sequencing and concordance between methods was 100%. Acute leukaemia patients, Hodgkin lymphoma patients and controls were genotyped using DNA extracted from peripheral blood or buccal swabs (98% of patients) or bone marrow (2% of patients). Breast cancer cases were genotyped using DNA extracted from paraffin embedded macrodissected non-tumour tissue.

Statistical analysis

Initial analysis for t-AML and breast cancer cases was conducted by comparing methylating agent exposed second cancer cases to second cancer cases with no prior exposure to

methylating agents. Further analysis for t-AML cases was performed using de novo AML cases as the reference population. Odds ratios (ORs) and 95% confidence intervals (CIs) were calculated using unconditional logistic regression,²⁰ with all analyses adjusted for age and sex. Where appropriate, further adjustments were made for cancer registry (breast cancer analysis). Given that radiotherapy exposure is strongly associated with breast cancer risk after Hodgkin lymphoma,¹⁷ and DNA MMR status may influence cellular response to ionising radiation, the analysis of second primary breast cancers was also adjusted for radiotherapy dose. All analyses were performed using Stata 9.1 1999 (Stata, College Station, Texas, USA).

RESULTS AND DISCUSSION

In order to test whether the *MLH1* –93 variant was associated with risk of t-AML in patients treated with methylating chemotherapy, we stratified the patient series (population 1) by prior exposure to these agents, and performed an analysis of exposed cases (n=12; all treated for primary Hodgkin lymphoma) versus non-exposed cases (n=69). Due to small numbers, this case:case comparison had low statistical power to detect anything other than a strong association. Nonetheless, we observed a significant association between the G>A *MLH1* –93 polymorphism and risk of t-AML after methylating chemotherapy. Specifically, carriers of the A-allele were significantly over-represented in the 12 t-AML cases whose prior exposure included a methylating chemotherapy agent (75.0%) compared to 69 t-AML cases without this exposure (30.4%) (OR for GA + AA vs GG: 5.49, 95% CI 1.18 to 25.60; p=0.03). Association with the *MLH1* –93 polymorphism was not seen when t-AML cases were stratified by any other major exposure group, including radiotherapy (30.0%), any type of chemotherapy (37.5%), DNA cross linking agents (46.4%), DNA topoisomerase inhibitors (47.1%) or anti-metabolite chemotherapy agents (20.0%).

In order to confirm that the observed association was specific to t-AML after exposure which included methylating chemotherapy, we genotyped 420 cases of de novo AML (population 2a) and 952 non-cancer controls (population 2b) (table 2). Carriers of the *MLH1* –93 A allele were significantly over-represented in t-AML cases with prior exposure to methylating agents (75.0%) compared to either de novo AML cases (36.9%) (OR for GA + AA vs GG: 5.31, 95% CI 1.40 to 20.15; table 2) or their matched controls (36.3%) (OR for GA + AA vs GG: 5.52, 95% CI 1.48 to 20.63). In contrast, there was no significant difference in *MLH1* –93 genotype distribution between de novo AML cases (36.9% carriers) and t-AML cases after chemotherapy per se (37.5% carriers) or radiotherapy alone (30.0% carriers) (table 2). There was also no significant difference in *MLH1* –93 genotype between de novo AML cases and their matched controls (OR for GA + AA vs GG: 1.03, 95% CI 0.80 to 1.31), or between primary Hodgkin lymphoma cases (population 4a) (34.7% carriers) and their matched controls (population 4b) (31.4% carriers) (OR for GA + AA vs GG: 1.16, 95% CI 0.78 to 1.75). These data suggest that the association seen is specific to t-AML after exposure which included methylating chemotherapy, and is not related to intrinsic risk of AML or Hodgkin lymphoma.

In addition to leukaemia, methylating agents can also cause solid tumours in animals, including mammary tumours.²¹ Given this, we determined *MLH1* polymorphism status in

women who developed breast cancer after treatment for Hodgkin lymphoma (population 3).¹⁷ Carrier frequency for the *MLH1* –93 G>A polymorphism was increased in 15 breast cancer cases with prior exposure to methylating chemotherapy (53.3%) compared to 22 with no methylating exposure (27.2%), with the difference of borderline statistical significance (OR for GA +AA vs GG: 4.01, 95% CI 0.83 to 19.35; $p=0.08$). These data, albeit based on small numbers, are consistent with the results observed for t-AML.

The –93 variant lies within a protein binding site and is one of three regions of the *MLH1* promoter required for maximal transcriptional activity.¹⁵ As such, it is plausible that polymorphic variation in this region affects *MLH1* expression. In support of this model, site directed mutagenesis of the adenine residue two bases downstream of the –93 variant (position –91) reduces promoter activity by 75%.¹⁵

Due to the rarity and small number of t-AML and second primary breast cancer cases in this study, the observed associations should be viewed circumspectly. Nevertheless, these data support the hypothesis that the –93 promoter variant of *MLH1* is associated with an increased risk of developing cancer after methylating chemotherapy for Hodgkin lymphoma. These results are especially important, since second primary cancers are now the leading cause of death among long term survivors of Hodgkin lymphoma.²² A future goal is to identify prospectively patient subgroups with a heightened susceptibility of developing therapy-associated second cancers in order to modify planned treatment approaches.²³ Thus, our finding adds to a growing body of research that will eventually facilitate the development of evidence based personalised medicine.

Acknowledgments

Funding: We acknowledge the support of the Medical Research Council DNA/RNA bank (University College London Hospital), a facility funded by the Kay Kendall Leukaemia Fund). The leukaemia work was supported by the Leukaemia Research Fund of the United Kingdom. The breast cancer work was supported in part by the Intramural Research Program of the United States National Cancer Institute, Division of Cancer Epidemiology and Genetics.

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Table 1

Description of therapy-related AML (t-AML) and breast cancer cases, de novo AML cases and controls, Hodgkin lymphoma cases and controls, by sex, age and prior therapy

Variable	Population 1 t-AML cases n(%)	Population 2a de novo AML cases n(%)	Population 2b de novo AML controls n(%)	Population 3 Second primary breast cancer cases n(%)	Population 4a Hodgkin lymphoma cas n(%)	Population 4b Hodgkin lymphoma controls n(%)
Total	96 (100)	420 (100)	952 (100)	37 (100)	242 (100)	225 (100)
Sex						
Male	39 (41)	221 (54)	509 (53)	na	149 (61.6)	138 (61.3)
Female	57 (59)	193 (46)	443 (47)	37 (100)	93 (38.4)	87 (38.7)
Age (years)						
Mean (range)	54.8 (17.5–77.B)	47.5 (16.2–70.0)	48.1 (16.3–69.8)	40.0 (28.0–54.0)	40.6 (16.3–67.3)	40.7 (16.3–65.5)
Prior therapy						
Radiotherapy only	40 (42)	na	na	22 (59)	na	na
Any chemotherapy*	56 (58)	na	na	15 (41)	na	na
Alkylating agents	28 (29)	na	na	15 (41)	na	na
Methylating agents	12 (12)	na	na	15 (41)	na	na
Topoisomerase inhibitors	17 (18)	na	na	nd	na	na
Anti-metabolites	14 (15)	na	na	nd	na	na
Tubulin inhibitors	16 (17)	na	na	nd	na	na
Corticosteroids	18 (19)	na	na	nd	na	na
Details not available	15 (16)	na	na	nd	na	na

na, not applicable; nd, data not collected.

* 35 of 56 t-AML cases and 14 of 15 second primary breast cancer cases had received both chemotherapy and radiotherapy. Alkylating agents: chlorambucil, dacarbazine, busulfan, mechlorethamine, procarbazine ifosfamide 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (CCNU), 1,3-bis (2-chloroethyl)-1-nitrosourea (BCNU), mitomycin C, any cisplatin derivative melphalan and cyclophosphamide. Methylating agents: procarbazine and dacarbazine. Topoisomerase inhibitors: mitoxantrone, adriamycin (doxorubicin), etoposide, epirubicin, idarubicin and daunorubicin (bleomycin is also included in this group). Anti-metabolites: methotrexate, azathioprine, 5-fluorouracil, cytosine arabinoside and hydroxyurea. Tubulin inhibitors: vincristine, vinblastine. Corticosteroids: prednisone and prednisolone (cyclosporin A is also included in this group). Cases are listed more than once if they received more than one type of therapy, thus the groups are not mutually exclusive.

Table 2

Number of cancer-free controls, therapy related and de novo AML cases, adjusted odds ratios (OR) and 95% confidence intervals (CI) for *MLH1* -93 by previous therapeutic exposure

<i>MLH1</i> -93 status	de novo AML (population 2A) (n = 420)	Controls (population 2B) (n = 952)	t-AML (population 1)					
			t-AML (%) (n = 96)	OR (95% CI)	Any chemotherapy (%) (n = 56)	OR (95% CI)	Methylating chemotherapy (%) (n = 12)	OR (95% CI)
GG	246 (63.1)	585 (63.7)	63 (65.6)	1	35 (62.5)	1	3 (25.0)	1
GA	128 (32.8)	292 (31.8)	30 (31.2)	0.91 (0.56 to 1.50)	18 (32.1)	0.99 (0.54 to 1.83)	7 (58.3)	4.63 (1.16 to 18.43)
AA	16 (4.1)	41 (4.5)	3 (3.1)	0.85 (0.23 to 3.08)	3 (5.4)	1.36 (0.37 to 4.93)	2 (16.7)	10.68 (1.61 to 71.04)
sna	30	34	–	–	–	–	–	–
GG	246 (63.1)	585 (63.7)	63 (65.6)	1	35 (62.5)	1	3 (25.0)	1
GA+AA	144 (36.9)	333 (36.3)	33 (34.4)	0.91 (0.56 to 1.47)	21 (37.5)	1.03 (0.58 to 1.85)	9 (75.0)	5.31 (1.40 to 20.15)
								0.61 (0.29 to 1.31)

sna, sample not amplified; t-AML, therapy related AML.

Odds ratios and 95% confidence intervals were estimated by logistic regression using de novo AML cases (population 2A) as the reference population and adjusting for sex and age at diagnosis.