Genetic and epigenetic variants in the *MTHFR* gene are not associated with non-Hodgkin lymphoma

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ABSTRACT

The methylenetetrahydrofolate reductase (*MTHFR*) gene codes for the MTHFR enzyme which plays a key role in the pathway of folate and methionine metabolism. Polymorphisms of genes in this pathway affect its regulation and have been linked to lymphoma. In this study we examined whether we could detect an association between two common non-synonymous *MTHFR* polymorphisms, 677C > T (rs1801133) and 1298A > C (rs1801131), and susceptibility to non-Hodgkin lymphoma (NHL) in an Australian case–control cohort. We found no significant differences between genotype or allele frequencies for either polymorphisms between lymphoma cases and controls. We also explored whether epigenetic modification of *MTHFR*, specifically DNA methylation of a CpG island in the *MTHFR* promoter region, is associated with NHL using blood samples from patients. No difference in methylation levels was detected between the case and control samples suggesting that although hypermethylation of *MTHFR* has been reported in tumour tissues, particularly in the diffuse large B-cell lymphoma subtype of NHL, methylation of this MTHFR promoter CpG island is not a suitable epigenetic biomarker for NHL diagnosis or prognosis in peripheral blood samples. Further studies into epigenetic variants could focus on genes that are robustly associated with NHL susceptibility.

1. Introduction

Non-Hodgkin lymphoma (NHL) is a diverse group of lymphoid proliferative disorders and makes up 90% of all solid tumours of the immune system (Shankland et al., 2012). The majority of the NHL tumour subtypes arise from the B lymphocytes and have been defined by the current fourth edition of the WHO classification of tumours of haemopoietic and lymphoid tissues (Swerdlow et al., 2008). The two most common subtypes are diffuse large B-cell lymphoma (DLBCL), which is rapidly aggressive, and the more indolent and slow progressing follicular lymphoma (FL) (Shankland et al., 2012). The highest rates of NHL are reported in the most economically developed countries such as the United States, Australia/New Zealand and northern Europe, and it predominantly affects white, non-hispanic men over 50 years of age (Morton et al., 2006).

During B lymphocyte development numerous mutations occur that can disrupt normal differentiation, proliferation and survival of these cells and thereby lead to NHL. Genetic lesions that are not lethal may be later modulated by environmental (i.e. virus or chemical), genetic polymorphisms or even epigenetic factors (e.g. DNA hypo- or hypermethylation) that would increase risk of NHL. Recent large genome-wide association studies (GWAS) have implicated single nucleotide polymorphisms (SNPs) at the human leukocyte antigen (HLA) region in both DLBCL and NHL, as well as a number of SNPs at non-HLA loci including transcription factors and oncogenes (Cerhan et al., 2014; Skibola et al., 2014). In previous studies gene variants in genes involved in DNA double-strand break and repair and the one-carbon metabolic pathway have been associated with NHL (Skibola et al., 2007).

The one-carbon metabolic pathway, also known as the folate and methionine metabolism pathway, is responsible for DNA synthesis and DNA methylation. MTHFR is the most important and widely studied enzyme within one-carbon (methyl group) metabolism which irreversibly catalyses the conversion of 5,10-methylenetetrahydrofolate to 5-methylTHF and is responsible for regulating the distribution of one-carbon moieties between the separate arms of DNA synthesis or methylation. These one-carbon transfer reactions are supported by different nutritional co-enzymes, such as vitamin B2, B6, B12, methionine and folate (Lim et al., 2007). Deficiencies of nutrients or genetic polymorphisms of the genes involved in this pathway have been associated with the onset of cancer through improper DNA synthesis and repair

as well as disrupted methylation and aberrant gene expression (Choi and Mason, 2000). Previous studies have reported that some genetic variants in this pathway are associated with NHL (Skibola et al., 2007; Lightfoot et al., 2005; Matsuo et al., 2001; Matsuo et al., 2004; Niclot et al., 2006; Lee et al., 2007; Kim et al., 2008). Two of the most well-studied common SNPs in the *MTHFR* gene are at nucleotide position 677, where there is a cytosine to thymine base change (rs1801133; 677C > T) and at nucleotide position 1298 where there is an adenine to cytosine base change (rs1801131; 1298A > C). Both polymorphisms result in a missense mutation conferring a protein conformational change with lowered enzyme activity (Robien and Ulrich, 2003; Chen et al., 2002). As well as polymorphisms which change the activity of MTHFR via changes in amino acid sequence, the level of expression of the protein may be modulated via polymorphisms that affect regulatory sequences or by epigenetic modifications.

Epigenetic modifications are changes to the genome, by mechanisms other than changing the underlying DNA sequence that can result in heritable modulation of gene expression or cellular phenotype. They include potentially reversible modifications such as DNA methylation, histone modification and miRNA regulation that in turn affect gene expression and protein translation. DNA methylation is one of the best studied epigenetic modifications which occurs when a methyl group is covalently added to the cytosine base ring at symmetric CG dinucleotides in the DNA sequence. CpG islands (CGIs) at promoter regions are protected from methylation to enable transcription and gene expression, while methylated CGIs are spread widely across the genome to silence transposons and retroviruses, but also genes by recruitment of methyl-binding proteins which participate in silencing gene transcription (Illingworth and Bird, 2009). Aberrant DNA methylation and deregulated gene expression is well established in NHL (Shaknovich and Melnick, 2011). Genetic variants or polymorphisms in genes that influence methylation may promote NHL by causing hypo- or hypermethylation of certain proto-oncogenes or tumour suppressor genes, respectively (Skibola et al., 2007). DNA methylation has many clinical applications in terms of subtype diagnosis and prognosis. It may not only serve as a tumour biomarker but also as a therapeutic target as it is potentially reversible with demethylating agents and methylation inhibitors (Lubbert, 2000).

A study by Pike et al. in 2008 evaluated the DNA methylation levels at over 500 CpG islands in DLBCL tumours and found that a *MTHFR* CpG island was one of the twelve that showed significant methylation in over 85% of the tumours (Pike et al., 2008). We have previously investigated the CpG island upstream of the *MTHFR* transcription start site and found that increased methylation was associated with risk of stroke, and furthermore this was detectable in blood DNA samples (Wei et al., 2014; Wei et al., 2015).

The aim of this study was to determine whether an association exists between polymorphisms and/or methylation of *MTHFR* and susceptibility to NHL. Previous studies assessing the gene promoter CpG island methylation status have used tumour cells and tissue as a DNA source. For this study only peripheral blood samples were available and thus the aim was to also assess whether blood is a useful source for methylation detection, which would potentially aid in a more rapid and less intrusive diagnostic testing.

2. Materials and methods

2.1. Sample collection and participants.

Patients diagnosed with NHL (cases) and healthy volunteers (controls) gave written consent to participate in this study and completed a detailed questionnaire. Case samples were collected from the Gold Coast Hospital, Princess Alexandra Hospital as well as Haematology and Oncology clinics around Australia. Ethics was approved for sample collection as part of the on-going Queensland University of Technology Lymphoma Project (1400000125) by the Princess Alexandra Hospital's Human Research Ethics Committee (HREC/06/QPAH/047). Peripheral blood was collected from case and control volunteers into ethylenediaminetetra acetic acid (EDTA) vacutainer tubes and sent to the Genomics Research Centre. Blood was stored at −20 °C on arrival and each sample was allocated a de-identifying number and barcode. Around 350 case samples were collected, but only 220 were suitable for analysis taking into account exclusion criteria including non-Caucasian ethnicity, unknown diagnosis or family history of haematological malignancy. Control samples (n = 210) were collected and these were matched to suitable cases according to age (+ or −5 years) and sex. Out of the 220 cases, 106 (48.4%) were male and 113 (51.6%) were female (one case was missing gender). Out of the 210 controls, 95 (45.2%) were male and 115 (54.8%) were female. The mean age of cases in 2013 was 63.72 years with a standard deviation (SD) of 12.95 years. The mean age of controls in 2013 was 63.14 years with a SD of 13.03 years.

2.2. DNA extraction and clean-up

Genomic DNA was extracted from peripheral blood leukocytes of all case and control samples by the traditional salting-out method (Miller et al., 1988). If required DNA was additionally cleaned up by phenol:chloroform extraction and ethanol precipitation. DNA quality and concentration was quantified using a Nanodrop spectrophotometer. Working DNA samples were prepared with a final concentration of 20 ng/μL and stored at 4 °C.

2.3. Genotyping

The following primers were used to genotype *MTHFR* polymorphisms:. *MTHFR*677C > T fwd: 5′-AGGACGGTGCGGTGAGAGTG-3′, *MTHFR*677C > T rev: 5′-TGAAGGAGAAGGTGTCTGCGGGA-3′, *MTHFR*1298A > C fwd: 5′-CACTTTGTGACCATTCCGGTTTG-3′, and *MTHFR*1298A > C rev: 5′-CTTTGGGGAGCTGAAGGACTACTAC-3′. For the 677C > T polymorphism, amplification was carried out by polymerase chain reaction (PCR) on a thermocycler instrument (Veriti 96 well Thermal Cycler, Applied Biosystems). Restriction Fragment Length Polymorphism (RFLP) was performed with overnight digestion with *Hinf*1 and the three genotypes were detected after agarose gel electrophoresis: CC (homozygous wild type, 198 bp), CT (heterozygote of 198 bp, 175 bp and 23 bp) and TT (homozygote mutant, 175 bp and 23 bp). The 1298A > C polymorphism was genotyped by high resolution melt (HRM) analysis on the Rotor-Gene Q (Qiagen) instrument with appropriate positive and negative controls. Amplification followed by a melting phase between 75 °C and 85 °C produced three different melting curves representing the three genotypes: homozygous wild type AA, heterozygote AC and homozygous mutant CC. Positive controls were confirmed by Sanger sequencing on a 3130 Genetic Analyser (Applied Biosystems).

2.4. Bisulfite conversion of genomic DNA and pyrosequencing

Bisulfite conversion of 500 ng of sample DNA was performed as a preliminary step to CpG methylation analysis using the EZ DNA Methylation-Gold™ Kit (Zymo Research) according to the manufacturer's instructions.

The PyroMark™ CpG Assay, Hs CLCN6_01_PM (QIAGEN), was used for the detection of methylation by PCR amplification of bisulfite treated DNA and pyrosequencing by the PyroMark™ Q24 instrument. The CpG island sequence of interest prior to bisulfite conversion (GGTCACTGAGTCAC**C**GATGGGGG**C**GAGGAYA**C**GGGC) contains 3 CpG sites where Y denotes a pyrimidine SNP, C or T. This CpG island is shared with the 5′ transcription start site of the *CLCN6* (chloride channel 6) gene which is transcribed in the opposite direction. Amplification of bisulfite converted DNA was performed by PCR with *MTHFR* primers, of which the reverse primer was biotinylated.

Biotinylated PCR product was immobilised to Sepharose beads, which were captured onto filter probes and after washing were released into the wells of the PyroMark™ Q24 Plate containing the MTHFR-specific sequencing primer. Samples were run on a QIAGEN Q24 pyrosequencer using PyroMark™ Q24 Gold reagents and the percent methylation levels determined at the three CpG sites in the assay using Pyromark Q24 software.

10 samples from the case group were randomly selected for a reproducibility test to determine reliability of the pyrosequencing assay. Mean CpG island methylation percentages were calculated and compared with mean reproducibility CpG island methylation percentages where a range of $^+/-2\%$ was acceptable. CpG1 was found to show little variation between three technical replicates, whereas methylation levels at CpG2 and CpG3 showed $>^+/-2\%$ variation and were not included in the analysis.

2.5. Data analysis

Statistical analysis of the data was carried out using SPSS v20.0 (SPSS Inc., Chicago, IL, USA). Genotype frequencies were examined for Hardy–Weinberg equilibrium in the 210 control subjects. Chi Square cross-tabulation analysis was performed to examine the association between the genotypes and alleles for each SNP and presence of NHL: the sample of subjects was classified as either "case" or "control" and associated with the different genotype and allele frequencies. Odds ratios and 95% confidence intervals were estimated using a binary logistic regression model for binary outcomes (1 = case, 0 = control) that controlled for gender and age. Unadjusted P values and odds ratios were calculated for each covariate. Adjusted P values and odds ratios were subsequently calculated by including all the covariates. Finally a stepwise forward conditional analysis was performed where statistical significance was assessed after the stepwise removal of each covariate individually. P values were calculated where a value of P < 0.05 was statistically significant. A study with a sample size of n = 200 each for cases and controls was estimated to have approximately 70% power to detect associations which increased NHL risk two-fold.

3. Results

3.1. Genotyping

The MTHFR 677C > T polymorphism was genotyped by RFLP in 220 cases and 210 controls. Of these 13 case and 9 control samples failed to amplify giving a genotyping success rate of > 94%. Genotypic frequencies were found to be in Hardy–Weinberg equilibrium for the control group (P = 0.393) with genotype numbers and frequencies in the case and control cohorts summarised in Table 1. χ^2 analysis showed that there were no significant difference between cases and controls for either genotypic or allelic frequencies (P = 0.445 and P = 0.95, respectively). The odds ratio (OR) and 95% confidence intervals (CI) for the allelic frequency was 0.99 (0.74–1.33) indicating no increased relative risk of NHL with presence of the 677C > T polymorphism.

The MTHFR 1298A > C polymorphism was genotyped by HRM in 220 cases and 210 controls, with 9 cases and 8 controls failing to amplify.

The control cohort genotype frequencies were found to be in Hardy–Weinberg equilibrium (P = 0.502) with genotype and allele frequencies for the case and control cohorts also shown in Table 1. There was no significant difference between genotypic and allelic frequencies of the case and control cohorts (P = 0.948 and P = 0.82, respectively). An OR of 1.03 (0.77–1.38) indicates no increased relative risk of NHL with the presence of the 1298A > C polymorphism.

These results show that there is no independent association between either of the MTHFR polymorphisms examined and NHL susceptibility in this population cohort.

3.2. MTHFR promoter methylation

We next quantified methylation at a CpG island of the MTHFR promoter in a subset of the NHL cases and controls. Cases were selected accordingly to include both NHL subtype, i.e. DLBCL and FL, and where ample stock DNA was available. Bisulfite conversion, amplification, and pyrosequencing were performed for 53 cases and 46 controls. Cases and controls were not specifically matched to each other however they were matched in general as for the genotyping assays. Age and sex covariates were controlled for in the logistic regression analysis. Methylation percentages were obtained for each of the three CpG sites in the sequence of interest and results were reported in the form of a Pyrogram™ and statistical report. As we found variation to be much greater at CpG2 and CpG3 in technical replicates, we restricted analysis to CpG site 1, however methylation was low at all sites (<5%).

Fig. 1 shows that these values were found to be within a normal distribution for cases and controls by frequency distribution analysis. One case outlier with a CpG1 percentage of 4.8% was included in the analysis. The mean CpG1 percentage for cases was 3.21% (SD 0.57) and for controls was 3.15% (SD 0.64). An independent sample T-test was performed on 29 DLBCL cases and 24 FL cases. There was no significant difference in methylation quantity between DLBCL and FL cases (p = 0.910), therefore subtypes were not considered for further analysis.

3.3. Logistic regression analysis

We performed a logistic model regression analysis using SPSS for case and control samples, including both MTHFR polymorphisms and methylation percentage of CpG1. We controlled for covariates of age and gender. In all analyses a p value of <0.05 was considered statistically significant. All odds ratios were calculated with 95% confidence interval limits. Unadjusted P values were calculated for each covariate individually. The P values and odds ratios for the covariates age, gender, 677C > T, 1298A > C and CpG1 are given in Table 2. Adjusted P values were calculated to determine significance of combined covariates and odds ratios were calculated and are shown in Table 2. A stepwise forward conditional method was used in the logistic regression model to determine significance of covariates after the stepwise removal of each covariate from the analysis. P values were calculated and are given in Table 2.

These results show that there was no significant difference between the individual or combined covariates or after stepwise removal of

Table 1

Genotype and allele frequencies of MTHFR 677C > T and 1298A > C in a non-Hodgkin lymphoma case–control population.

SNP	Group	Genotypes			P	Alleles		P
rs1801133		CC (%)	CT (%)	TT (%)		C (%)	T (%)	
677C > T	Control	88 (43.8)	94 (46.8)	19 (9.5)		270 (67.2)	132 (32.8)	
	Case	97 (46.9)	85 (41.1)	25 (12.1)	0.445	279 (67.4)	135 (32.6)	0.945
		AA (%)	AC (%)	CC (%)		A (%)	C (%)	
rs1801131	Control	93 (46.0)	85 (42.1)	24 (11.9)		271 (67.1)	133 (32.9)	
1298A > C	Case	94 (44.5)	92 (43.6)	25 (11.8)	0.948	142 (33.6)	133 (32.9)	0.824

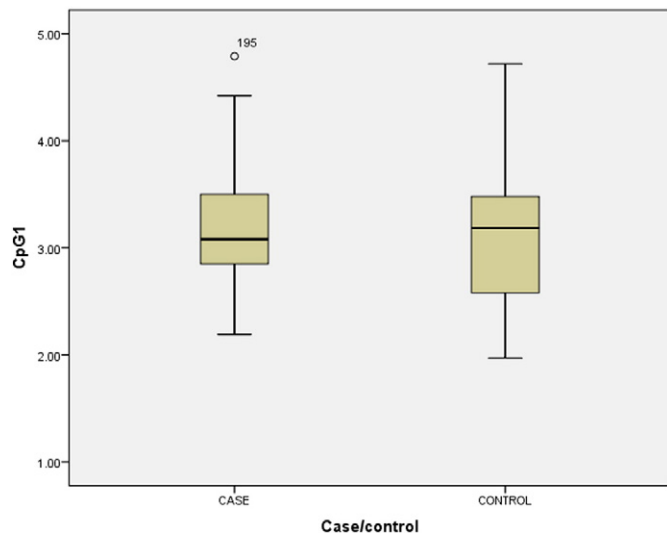P values were calculated by χ^2-analysis, significance is taken at P ≤ 0.05.

Fig. 1. Frequency distribution analysis of CpG1 methylation. A stem-and-leaf plot showing distribution from the mean in cases (3.21%, SD 0.57) and controls (3.15%, SD 0.64).

covariates in cases and controls and therefore no increased risk or associated susceptibility to NHL.

4. Discussion

A number of case–control studies of different geographical and ethnic populations on the association of *MTHFR* polymorphisms with NHL susceptibility have produced conflicting results, with some showing reduced risk (Matsuo et al., 2001; Matsuo et al., 2004; Kim et al., 2008), some showing increased risk (Skibola et al., 2007; Niclot et al., 2006; Lee et al., 2007; Siraj et al., 2007; Ismail et al., 2009; Nasr et al., 2012) and some showing no risk (Lightfoot et al., 2005; Gemmati et al., 2004; Deligezer et al., 2006; Weiner et al., 2011). In this study we found no association between either the 677C > T or the 1298A > C polymorphisms and susceptibility to NHL in a relatively small Australian Caucasian population, which is concordant with three previous studies performed on Caucasians, namely Russian, Italian and British populations. Our data do not correlate with a previous Australian study, however this study showed only a marginally increased risk with 677C > T and NHL and included subjects of Asian descent (Lee et al., 2007). Furthermore, it should also be noted that because of our small sample size the study would be unable to significantly detect odds ratios below 2. There may be a differential distribution of the MTHFR 677TT genotype between cases and controls (12.1% vs 9.5%), but insufficient power means that it is not detected as significant. Genetic predisposition with respect to genetic variants may also depend on subtype, population, geographical region, nutritional status and environmental factors. We did not analyse polymorphisms according to the different NHL subtypes due to the limited sample size.

Table 2
Logistic regression analysis for unadjusted and adjusted P values and odds ratios for covariates between cases and controls.

Covariate	Unadjusted P	Unadjusted OR (95% CI)	Adjusted P	Adjusted OR (95% CI)
Age	0.574	0.999 (0.996–1.002)	0.143	1.020 (0.993–1.047)
Gender	0.834	0.987 (0.878–1.111)	0.491	0.747 (0.326–1.712)
677 > T	0.803	0.986 (0.884–1.100)	0.580	1.196 (0.634–2.257)
1298A > C	0.622	0.973 (0.874–1.084)	0.946	0.979 (0.526–1.821)
CpG1	0.438	0.953 (0.843–1.077)	0.262	0.685 (0.353–1.328)

P values were calculated by χ^2-analysis, significance is taken at $P \leq 0.05$.

Deficiencies of nutrients or genetic polymorphisms of the genes involved in the folate pathway have been associated with the onset of cancer through improper DNA synthesis and repair as well as disrupted methylation and aberrant gene expression (Choi and Mason, 2000). *MTHFR* polymorphisms reduce activity of the MTHFR enzyme and the resulting loss of methyl groups can potentially induce DNA hypomethylation in blood leukocytes (Stern et al., 2000; Friso et al., 2002), as well as CGI promoter methylation or CpG island methylator phenotype (CIMP) in colorectal cancer (Oyama et al., 2004; Rustgi, 2007). For our study we decided to evaluate methylation status of the *MTHFR* promoter to determine whether it influences NHL risk and whether there is a potential association with the common *MTHFR* polymorphisms in NHL cases. However, our statistical analyses of *MTHFR* CpG island methylation status both independently (Fig. 1), and in correlation with the presence of *MTHFR* polymorphisms (Table 2), showed no associations with susceptibility to NHL.

Although Pike et al. (2008) found methylation of *MTHFR* CpG islands in 85% of DLBCL tumours (Pike et al., 2008), we did not detect hypermethylation of *MTHFR* in blood in this study. Methylation-enriched genome-wide bisulfite sequencing of an FL cell line also did not find hypermethylation of *MTHFR* (Choi et al., 2010). An independent sample T-test performed on both DLBCL and FL samples showed no significant difference in methylation between the two groups and the *MTHFR* CpG island analysed in this study was essentially unmethylated in all individuals. Therefore while *MTHFR* CpG islands may be methylated in tumour tissue, particularly in DLBCL, this gene does not appear to be useful as a biomarker for NHL risk in blood samples. A caveat to this result is that we only investigated one CpG island, which we had previously identified as increasing stroke risk (Wei et al., 2014; Wei et al., 2015). Transcription of *MTHFR* is complicated, with multiple transcriptional start sites and isoforms, so that other CpG islands or regions may be important depending on disease context. For example, the frequency of *MTHFR* promoter methylation was found to be increased in patients with end-stage renal disease compared to controls and this was detectable in peripheral blood samples (Ghattas et al., 2014). A review by Li et al. (2012) concluded that blood-based DNA methylation patterns are potentially useful as cancer biomarkers, although further studies need to be carried out (Li et al., 2012). Future epigenetic studies may be warranted in genes that have been robustly implicated in NHL, such as the HLA loci, as there is evidence for methylation of HLA genes in a range of conditions, including some in which the differences are detectable in blood (Graves et al., 2013; Adams et al., 2014; Hong et al., 2015).

In conclusion we have shown that the 677C > T and 1298A > C polymorphisms are not associated with susceptibility to NHL and that a *MTHFR* promoter region CpG island is not influenced by epigenetic DNA methylation in peripheral blood leukocytes in our Australian cohort of NHL patients.

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