UNIVERSITY OF THESSALY

SCHOOL OF MEDICINE

LABORATORY OF BIOMATHEMATICS

M.SC. "RESEARCH METHODOLOGY IN BIOMEDICINE, BIOSTATISTICS AND CLINICAL BIOINFORMATICS

MASTER THESIS

THE QUALITY OF REPORTING OF GENETIC ASSOCIATION STUDIES FOR MTHFR (rs 1801131) IN CHRONIC LYMPHOCYTIC LEUKEMIA USING THE STREGA STATEMENT AND META-ANALYSIS

STAVROULA OIKONOMOU

Committee members

Prof. Stefanidis Ioannis

Prof. Zintzaras Elias

Prof. Hadjichristodoulou Christos

LARISSA, AUGUST 2015

THE QUALITY OF REPORTING OF GENETIC ASSOCIATION STUDIES FOR MTHFR (rs 1801131) IN CHRONIC LYMPHOCYTIC LEUKEMIA USING THE STREGA STATEMENT AND META-ANALYSIS

STAUROULA OIKONOMOU

Faculty of Medicine, Laboratory of biomathematics

Abstract

Background: With the genetic studies having become very popular in the last years, the need for the creation of specific checklists, which help the authors to improve the way they write articles, has arisen. A large number of genetic studies focus on the MTHFR gene and its two single nucleotide polymorphisms (SNPs), (C677T) and (A1298C), trying to find an association between the aforementioned gene and Chronic Lymphocytic Leukemia.

Aim: The purpose of the present study is to evaluate the quality of reporting of genetic association studies for MTHFR (rs 1801131) in Chronic Lymphocytic Leukemia using the STREGA statement and to perform a Meta-analysis, in order to explore the association between MTHFR (rs1801131) polymorphism and CLL/SLL risk.

Methods: All of the studies, published before July 2015, were identified through computer based searches of the following databases, PubMed and HuGE. The articles were evaluated based on the STREGA statement and a Meta-analysis was performed using the ORG and the "ORGGASMA" software.

Results: The results of the evaluation of quality reporting, based on STREGA statement were from highest to lowest: 17.04/22 for Lim et al. 2006, 14.41/22 for Nuckel et al. 2004, 11.35/22 for Rudd et al. 2004 and for Gra et al. 2007, 7.76/22. As far as the Meta-analysis is concerned, the p-value for the Q-statistic is 0.48, which is more than 0.10, so there is no significant heterogeneity between the studies. For this reason the Fixed effects model was selected, for MTHFR A1298C and ORG was found to be 0.9849 with 95% Confidence Interval (0.883-1.098). The 95% CI includes the value "1", so as a result the ORG is not considered significant.

Conclusion: Genetic articles appear to have methodological errors that could be avoided with the use of the recommendations given in STREGA statement. In the Meta-analysis, the MTHFR (rs1801131) polymorphism was not significantly associated with the risk for CLL/SLL,

Keywords: STREGA, MTHFR, A1298C, SLL, CLL, chronic lymphocytic leukemia, polymorphism, meta-analysis, genes.

TABLE OF CONTENTS

1. INTRODUTION 3

1.1 Chronic lymphocytic leukemia 3

1.2 MTHFR gene 4

1.3 STrengthening the REporting of Genetic Association studies Statement 5

AIM OF THE STUDY 5

2. MATERIALS AND METHODS 6

2.1 Literature search 6

2.2 Inclusion criteria 6

2.3 Study selection 6

2.4 Data Extraction 6

2.5 Quality of reporting of genetic associations studies 7

2.6 Statistical Analysis 7

3. RESULTS 8

3.1 Published articles. 8

3.2 Summary of quality of reporting 9

3.3 Summary of study characteristics 10

3.4 Association between MTHFR A1298C and CLL/SLL risk 10

4. DISCUSSION 11

5. REFFERENCES 15

6. APPENDICES 17

1. INTRODUTION

1.1 Chronic lymphocytic leukemia

Cancer is considered to be one of the most challenging public health issues. It is estimated that the cancer cases and cancer deaths in 2008 were nearly 12.7 million and 7.6 million worldwide respectively (He et al. 2014). Leukemias are defined as a group of cancers deriving from hematopoietic cell lines. Disruptions of the normal function of the genes at these locations can cause an alteration from normal blood cell development. This can be a result of genetic translocations, inversions, or deletions in hematopoietic cells (Robien et al. 2003).

The mechanism, by which leukemias cause disease, includes the accumulation of dysfunctional or non-differentiated leukemic cells, in the bone marrow. These cells slowly replace normal hematopoietic cells, causing signs and symptoms such as anemia, fatigue, bleeding, and infections (Robien et al. 2003). They can derive from myeloid or lymphoid cell lines, or both. The latter is the case in myeloid/lymphoid or mixed-lineage leukemia (MLL) (Rudd et al. 2004).

Leukemias are categorized, depending on how fast disease is progressing, as either acute or chronic. There are four major forms of leukemia. A) Acute Lymphocytic Leukemia (ALL), B) Acute Myelogenous Leukemia (AML), C) Chronic Lymphocytic Leukemia (CLL), and D) Chronic Myelogenous Leukemia (CML) (Robien et al. 2003).

The most common is considered to be chronic lymphocytic leukemia (CLL) (Rudd et al. 2004), which is categorized as a B-cell malignant lymphoma. The prognosis can be anywhere from good to bad. It tends to target more men than women, with the median age of patients at diagnosis at 65 years (Rozman et al. 1995).

CLL and small lymphocytic lymphoma (SLL) are considered to be the same disease at different stages, sharing the same immunophenotype and similar clinical courses (Zintzaras et al. 2009).

CLL is considered to be an idiopathic condition. Despite the existence of evidence supporting a genetic background of the disease, no specific gene has been associated with it (Rozman et al. 1995) (Kalil et al. 1999).

Recently there have been more publications investigating that association. This increase can be explained if one considers how easily accessible genetic data have become and how much genotyping technology has developed (Zintzaras et al. 2009). Still, the evidence remains insufficient, not only due to the use of small study samples but also due to the heterogeneity of the populations. (Zintzaras et al. 2009)

1.2 MTHFR gene

Methylenetetrahydrofolate reductase (MTHFR) is an enzyme, whose main function is the catalyzation of redundant methylenetetrahydrofolate to methyltetrahydrofolate, which in turn plays part in the methylation of homocysteine to methionine (Goyette et al. 1994). The location of the gene for MTHFR (EC 1.5.1.20) is at the end of the short arm of chromosome 1 (1p36.3) (Figure 1).

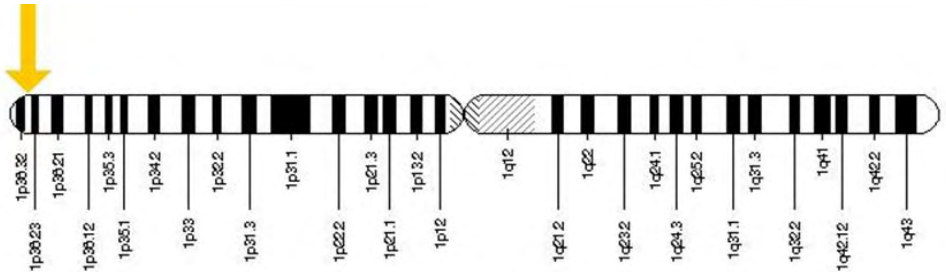


Figure 1. Location of MTHFR (http://ghr.nlm.nih.gov/gene/MTHFR)

Folate plays a part in the conversion of homocysteine to methionine and in purine and pyrimidine synthesis. It acts as a carrier for single carbon fragments. Its primary form is 5-methyl-THF. The reduction of 5,10-methylene tetrahydrofolate (THF) can be catalysed in 5-methyl-THF, irreversibly by the 5,10 methylenetetrahydrofolate reductase (MTHFR) enzyme. Therefore, any decline in the activity of MTHFR results in an increase of 5 methylenetetrahydrofolate, which in turn results in transfer of a methyl group converting dUMP to dTMP. This conversion causes uracil to be ill-placed into the DNA, which is claimed to have carcinogenic effects, such as DNA double-strand breaks. (Rudd et al.2004) Methylation, as the conversion of dUMP to dTMP is named, has been associated with a variety of types of cancers.

There are two single nucleotide polymorphisms (SNPs) that appear to tamper with MTHFR activity. The first appears in position 677 and is a C to T nucleotide transition (C677T) and the second in position 1298 and is an A to C transition (A1298C). (Gemmati et al 2007) The second polymorphism (A1298C) causes the replacement of glutamine to alanine at codon 49 (Robien et al. 2003). Some studies support that A1298C has an impact on MTHFR in homogenous individuals (Rudd et al.2004).

In the present study, the MTHFR (rs 1801131) polymorphism was used. There are 2 alleles for the present polymorphism genetic variant, so there are the following genotypes AA, AC, CC.

1.3 STrengthening the REporting of Genetic Association studies Statement

The number of genetic studies that are being published has experienced a substantial increase over the last years (Little et al. 2009). Various checklists and recommendations have been developed, in order to improve the quality of studies (Vandenbroucke et al. 2009), such as the STrengthening the Reporting of OBservational Studies in Epidemiology (STROBE) Statement for cross-sectional, case-control and cohort studies. For the sole purpose of focusing on the quality of genetic studies, an extension of the STROBE Statement called the STrengthening the REporting of Genetic Association studies (STREGA) Statement has been developed. The STREGA statement has been improved with revisions on 12 of the 22 items of the STROBE statement (Little et al. 2009).

Genetic studies have their distinct challenges that differentiate them from other observational studies. These include complex gene pathways, gene-environment and gene-gene interactions. Many studies even suffer from methodological issues, such as inadequate reporting of results (Little et al. 2009).

In order to overcome these methodological problems the STREGA statement revisions focus on five main topics. A) Genotyping errors B) population stratification C) modeling haplotype variation D) Hardy-Weinberg equilibrium (HWE) and E) replication (Little et al. 2009).

AIM OF THE STUDY

The purpose of the present study is to evaluate the quality of reporting of genetic association studies for MTHFR (rs 1801131) in Chronic Lymphocytic Leukemia using the STREGA statement and to perform a Meta-analysis, in order to explore the association between MTHFR (rs1801131) polymorphism and CLL/SLL risk.

2. MATERIALS AND METHODS

2.1 Literature search

All of the studies, published before July 2015, were identified through computer based searches of the following databases, PubMed and HuGE, using the following terms: “chronic lymphocytic leukemia”, “CLL”, “chronic lymphocytic leukaemia”, “chronic lymphoid leukemia”, “chronic lymphoid leukaemia”, “small lymphocytic lymphoma”, “SLL”, “lymphocytic lymphoma”, “gene”, “polymorphism”.

2.2 Inclusion criteria.

The studies included had to satisfy the following criteria:

1) Evaluate MTHFR A1298C polymorphisms
2) Include cases of chronic lymphocytic leukemia
3) Are either case-control study, nested case-control study or a cohort study
4) Are written in English

Furthermore, for the studies to be included in the Meta-analysis, they had to satisfy an additional set of criteria. The studies had to provide all the numbers of cases and controls at each genotype and be of case control design exclusively.

2.3 Study selection

All of the articles were screened by title or abstract. If the inclusion criteria were satisfied, the article was used in the present study. Some studies used patients with different types of Non-Hodgkin Lymphoma. Those studies were screened by reading the ‘materials and methods’ section of the papers. If there were patients with CLL/SLL, they were included.

2.4 Data Extraction

The data from every paper, which was relative to the subject, were summarized into a table. The table included all of the important information about the studies, such as the first author’s name, the publication year, the mean age and the range of age, the genotyping method, the ethnicity and the number of cases and controls. Moreover a second table, which has the frequencies of genotypes, is also provided.

2.5 Quality of reporting of genetic associations studies

As stated above, the STREGA statement (as an extension of STROBE for genetic studies) was used to evaluate the quality of reporting of genetic associations studies for MTHFR (rs 1801131) in Chronic Lymphocytic Leukemia. The STREGA statement was used as Little et al. 2009 described and recommended (Little et al. 2009).

2.6 Statistical Analysis

The main purpose of the present study was to evaluate the association between MTHFR (rs 1801131) polymorphism and CLL/SLL risk. Using the Chi-square test, it was tested, if the control's genotype distribution was in Hardy Weinberg equilibrium (HWE). For the Meta-analysis of association of MTHFR polymorphism and CLL/SLL risk, an alternative methodology was performed, called the Generalized Odds Ratio (ORG). The ORG is a genetic model-free approach, equal to the probability of being diseased divided by the probability of being non-diseased (Zintzaras.2010). "ORGGASMA" software, which is available at http://biomath.med.uth.gr, was used to calculate the ORG and 95% Confidence Interval using either the Fixed effects model (FE) or the Random effects model, depending on heterogeneity (Zintzaras.2010). Q and I^2 were used to test for homogeneity in ORGGASMA. The present study used only the Recessive model (Liao et al. 2013) to calculate the OR, the variance of log(OR) known as theta and the 95% CI. The results were presented graphically using a forest plot. Publication bias was evaluated with a funnel plot and Egger's test (Lee.2015). All statistical analyses were performed using the SPSS version 21 software.

3. RESULTS

3.1 Published articles.

The purpose of the present study was to investigate the quality of reporting of genetic association studies for MTHFR (rs1801131) in chronic lymphocytic leukemia using the STREGA statement. In July 2015, a search in PubMed and HuGE databases was conducted. The search resulted in 52 articles being identified. However, only a few of them used patients with CLL/SLL and investigated the MTHFR polymorphism of interest (rs1801131). As a result only 4 of them were relative to the study and were included in the Meta-analysis.

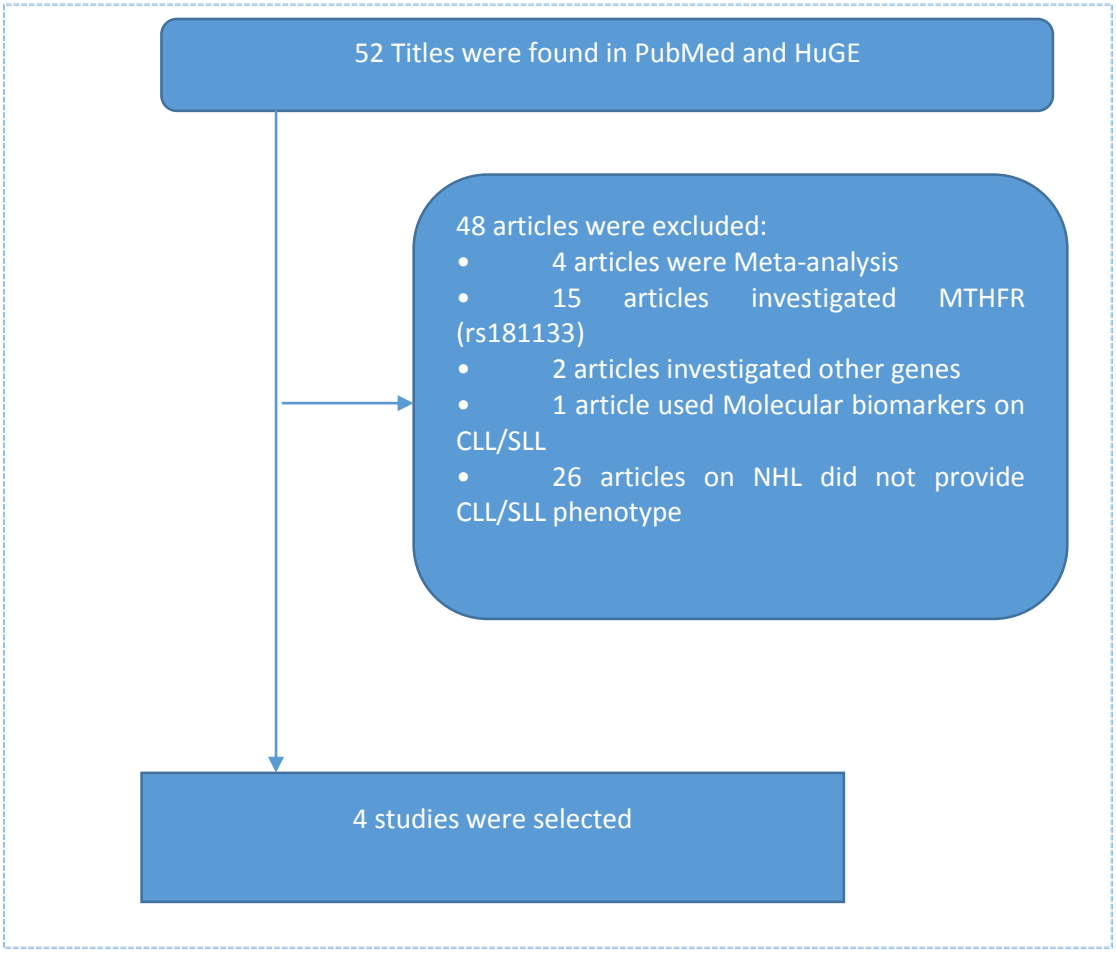


Figure 2: Flow chart of published articles retrieved for and excluded from an assessment of MTHFR of Chronic lymphocytic leukemia.

Table 3.1.1: Selected Studies

Author and Year	Number of study	Reason
Gra et al. 2007	1	CLL & MHTFR A1298C
Lim et al. 2006	2	
Rudd et al. 2004	3	
Nuckel et al. 2004	4	

3.2 Summary of quality of reporting

A table with all of the STREGA statement recommendations and the positive scoring by article is provided (Table 3.2.1). Every positive answer is equal to one point and every negative is equal to zero. For some of the recommendations that have subqueries, the 1 point is divided by the number of the respective subqueries, but the score of every item is calculated by the sum of all of its subqueries' answers.

TABLE 3.2.1. STREGA reporting recommendations, extended from STROBE Statement and the recording of quality of reporting of genetic associations studies for MTHFR (A1298C) in CLL.

Item	Item number	STROBE Guideline	Extension for Genetic Association Studies (STREGA)	Gra et al. 2007	Rudd et al. 2004	Nuckel et al. 2004	Lim et al. 2006	Total
Title and Abstract	1	(a) Indicate the study's design with a commonly used term in the title or the abstract.		NO	NO	NO	YES	1*
		(b) Provide in the abstract an informative and balanced summary of what was done and what was found.		YES	YES	YES	YES	4*
				0.5™	0.5™	0.5™	1™	
Title and Abstract Total Score				0.5®	0.5®	0.5®	1®	
Introduction								
Background rationale	2	Explain the scientific background and rationale for the investigation being reported.		YES	YES	YES	YES	4*
Objectives	3	State specific objectives, including any pre-specified hypotheses.	*State if the study is the first report of a genetic association, a replication effort, or both.*	NO	NO	NO	YES	1*
Introduction Total				1®	1®	1®	2®	
Methods								

Item	Item number	STROBE Guideline	Extension for Genetic Association Studies (STREGA)	Gra et al. 2007	Rudd et al. 2004	Nuckel et al. 2004	Lim et al. 2006	Total
Study design	4	Present key elements of study design early in the paper.		NO	YES	NO	YES	2*
Setting	5	Describe the setting, locations and relevant dates, including periods of recruitment, exposure, follow-up, and data collection.		YES	YES	YES	YES	4*
Participants	6	Cohort study – Give the eligibility criteria, and the sources and methods of selection of participants. Describe methods of follow-up. Case-control study – Give the eligibility criteria, and the sources and methods of case ascertainment and control selection. Give the rationale for the choice of cases and controls. Cross-sectional study – Give the eligibility criteria, and the sources and methods of selection of participants.	*Give information on the criteria and methods for selection of subsets of participants from a larger study, when relevant*.	NO	NO	YES	YES	2*

		Cohort study – For matched studies, give matching criteria and number of exposed and unexposed. Case-control study – For matched studies, give matching criteria and the number of controls per case.						
Variables	7	*(a)* Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable.	*(b) Clearly define genetic exposures (genetic variants) using a widely-used nomenclature system. Identify variables likely to be associated with population stratification (confounding by ethnic origin).*	NO YES	YES YES	YES YES	YES YES	3* 4*
				0.5™	1™	1™	1™	
Data sources measurement	8*	*(a)* For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group.	*(b) Describe laboratory methods, including source and storage of DNA, genotyping methods and platforms (including the allele calling algorithm used, and its version), error rates and call rates. State the laboratory/centre where genotyping was done. Describe comparability of laboratory methods if there is more than one group. Specify whether genotypes were assigned using all of the data from the study simultaneously or in smaller batches.*	YES NO	YES YES	YES YES	YES YES	4* 3*

				0.5™	1™	1™	1™	
Bias	9	*(a)* Describe any efforts to address potential sources of bias.	*(b) For quantitative outcome variables, specify if any investigation of potential bias resulting from pharmacotherapy was undertaken. If relevant, describe the nature and magnitude of the potential bias, and explain what approach was used to deal with this.*	NO NO	NO NO	NO NO	NO NO	0* 0*
				0™	0™	0™	0.5™	
Study size	10	Explain how the study size was arrived at.		NO	NO	NO	NO	0*
Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen, and why.	*If applicable, describe how effects of treatment were dealt with.*	NO	NO	YES	NO	1*

Statistical methods	12	(a) Describe all statistical methods, including those used to control for confounding.	*State software version used and options (or settings) chosen.*	NO	YES	YES	YES	3*
		(b) Describe any methods used to examine subgroups and interactions.		NO	YES	YES	YES	4*
		(c) Explain how missing data were addressed.		NO	NO	NO	YES	1*
		Cohort study – If applicable, explain how loss to follow-up was addressed. Case-control study – If applicable, explain how matching of cases and controls was addressed. Cross-sectional study – If applicable, describe analytical methods taking account of sampling strategy.		NO	YES	YES	YES	3*
		(e) Describe any sensitivity analyses.		YES	NO	NO	NO	1*
			(f) State whether Hardy-Weinberg equilibrium was considered and, if so, how.	NO	YES	YES	YES	3*
			(g) Describe any methods used for inferring genotypes or haplotypes.	NO	YES	YES	YES	3*
			(h) Describe any methods used to assess or address population stratification.	NO	YES	NO	NO	1*
			(i) Describe any methods used to address multiple comparisons or to control risk of false positive findings.	NO	NO	NO	YES	1*

			(j) Describe any methods used to address and correct for relatedness among subjects	NO	NO	NO	YES	*1**
				0.1™	0.6™	0.5™	0.8™	
Material and Methods Total Score				2.1®	5.1®	5.5®	6.3®	
Results								
Participants	13*	Report the numbers of individuals at each stage of the study – e.g., numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed.	*Report numbers of individuals in whom genotyping was attempted and numbers of individuals in whom genotyping was successful.*	NO	NO	NO	YES	1*
		(b) Give reasons for non-participation at each stage.		NO	NO	NO	NO	0*
		(c) Consider use of a flow diagram.		NO	NO	NO	NO	0*
				0™	0™	0™	0.33™	
Descriptive data	14*	(a) Give characteristics of study participants (e.g., demographic, clinical, social) and information on exposures and potential confounders.	*Consider giving information by genotype.*	YES	YES	YES	YES	4*
		(b) Indicate the number of participants with missing data for each variable of interest.		YES	NO	NO	NO	0*

		Cohort study – Summarize follow-up time, e.g. average and total amount.		-	-	-	-	-
				0.5™	0.5™	0.5™	0.5™	
Outcome data	15 *	Cohort study-Report numbers of outcome events or summary measures over time.	*Report outcomes (phenotypes) for each genotype category over time*					
		Case-control study – Report numbers in each exposure category, or summary measures of exposure.	*Report numbers in each genotype category*	YES	YES	YES	YES	4*
		Cross-sectional study – Report numbers of outcome events or summary measures.	*Report outcomes (phenotypes) for each genotype category*					
Main results	16	(a) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (e.g., 95% confidence intervals). Make clear which confounders were adjusted for and why they were included.		NO	YES	NO	YES	2*
		(b) Report category boundaries when continuous variables were categorized.		NO	NO	NO	YES	1*
		(c) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period.		NO	NO	NO	NO	0
			(d) Report results of any adjustments for multiple comparisons.	NO	NO	YES	NO	1*
				0™	0.25™	0.25™	0.25™	

Other analyses	17	Report other analyses done – e.g., analyses of subgroups and interactions, and sensitivity analyses.		YES	YES	YES	YES	4*
			(b) If numerous genetic exposures (genetic variants) were examined, summarize results from all analyses undertaken.	YES	YES	YES	YES	4*
			(c) If detailed results are available elsewhere, state how they can be accessed.	NO	NO	NO	YES	1*
				0.66™	0.66™	0.66™	1™	
Results Total Score				2.16®	2.75®	2.41®	2.74®	
Discussion								
Key results	18	Summarize key results with reference to study objectives.		YES	YES	YES	YES	4*
Limitations	19	Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias.		NO	NO	YES	YES	2*
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations,		YES	YES	YES	YES	4*

		multiplicity of analyses, results from similar studies, and other relevant evidence.						
General izability	21	Discuss the generalizability (external validity) of the study results.		NO	NO	YES	YES	2*
Other Information								
Fundin g	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based.		NO	NO	YES	YES	2*
Discussion Total score				2®	2®	5®	5®	
Total score				7.76¥	11.35¥	14.41¥	17.04¥	

* Give information separately for cases and controls in case-control studies and, if applicable, for exposed and unexposed groups in cohort and cross-sectional studies.

* A positive answer erns on point and a zero for a negative answer. Then summarized all the numbers (0,1) an the line.

® A positive answer erns on point and a zero for a negative answer. Then summarized all the numbers (0,1) an the column.

¥ Summarized all the total score from Title and Abstract, Materials and Methods, Results and Discussion.

™ The sum of the answer from subqueries. A positive answer erns on point and a zero for a negative answer.

By section:

- **Title or Abstract and Introduction**: Lim et al. 2006 scored higher, having more positive points than the others. Nuckel et al. 2004, Rudd et al. 2004 and Gra et al. 2007 all did not provide a common term for the study design.

- **Materials and Methods**: Lim et al. 2006 scored better than the others with 6.3/9. They had more information about the study design and the methods. Nuckel et al. 2004 and Rudd et al. 2004 were at the same level scoring 5.5/9 and 5.1/9 respectively. But Gra et al. 2007 did not provide very detailed information concerning the patient sample and the statistical analysis, therefore the score was only 2.1/9.

- **Results**: All the articles were at the same level. Their scores were between 2.16/5 and 2.75/5. Gra et al. 2007 scored the lowest (2.16). Nuckel et al. 2004 followed with 2.41/5. Rudd et al. 2004 and Lim et al. 2006 scored 2.75 and 2.74/5 respectively.

- **Discussion**: Nuckel et al. 2004 and Lim et al. 2006 had a perfect score of 5/5, as opposed to the other two studies that scored 2/5.

The total scores, from highest to lowest, were: 17.04/22 for Lim et al. 2006, 14.41/22 for Nuckel et al. 2004, 11.35/22 for Rudd et al. 2004 and for Gra et al. 2007 7.76/22.

3.3 Summary of study characteristics

Table 3.2.2 summarizes all of the study design characteristics including country, ethnicity, genotyping technology, sample size, percentage of males, mean age or range of age and conclusion by article. The study design of all of the four studies was diagnostic case-control, with the cases being patients who had already been diagnosed with CLL/SLL. The ethnicity was mainly Caucasian, except for one study, in which it was mixed. The sample size ranged from 111 to 1141 and 32 to 949 for cases and controls respectively. TaqMan is used as a genotyping method in 2 out of 4 the studies. In one study (Nuckel et al. 2004) no information about the average age or gender of patients or controls was provided.

Table 3.2.2: Summary of studies characteristics.

Authors and year	Country	Study Design	Ethnicity	Genotyping Method	Mean Age	Males	Sample Size
Gra et al. 2007	Russia	Case Control	Caucasian	Hybrridization	53 (31-83)	57.8%	159 cases 177 controls
Lim et al. 2006	USA	Case Control	Mixed	Taqman	(20-74)	55% cases 53% controls	1141cases 949 controls
Rudd et al. 2004	UK	Case Control	Caucasian	Taqman	61 (21-97)& 59(26-93)& 60(21-91)	69.3%	832 cases 886 controls
Nuckel et al. 2004	Germany	Case Control	Caucasian	PCR-RFLP	-	-	111 cases 32 controls

3.4 Association between MTHFR A1298C and CLL/SLL risk

Table 3.4.1: Hardy –Weinberg equilibrium

Authors	Controls			X^2	p-value
	AA	AC	CC		
Gra et al. 2007	81	82	14	1.16	0.27
Lim et al. 2006	461	393	81	0.135	0.83
Nuckel et al. 2004	55	40	7	0.029	0.94
Rudd et al. 2004	412	389	85	0.4	0.62

All the controls distribution were in line with the Hardy-Weinberg equilibrium.

Table 3.4.2: Frequencies of MTHFR A1298C polymorphism.

Authors	Cases CLL/SLL				Controls				X^2	p-value
	AA	AC	CC	ALL	AA	AC	CC	ALL		
Gra et al. 2007	39	38	6	83	81	82	14	177	0.057	0.97
Rudd et al. 2004	397	363	72	832	412	389	85	886	7.557	0.75
Lim et al. 2006	540	480	104	1124	461	393	81	935	0.419	0.81
Nuckel et al. 2004	51	48	12	111	55	40	7	102	1.817	0.4

The frequency distribution of MTHFR A1298C polymorphism genotype, the result from Chi-square (X^2), p-value and the degree freedoms are shown in Table 2.3.2. All the p-value were not less than the 10% point of the x^2 distribution with 2 df and as a result none of the studies indicated statistically significant association.

If Q is less than the 10% point of the x^2 distribution, with 4-1=3 df, there is no significant heterogeneity across studies. In more detail, the results were Q= 2.47 with p-value = 0.48 and I^2= 0.0. Overall, the p-value for the Q-statistic is 0.48, which is more than 0.10, so there is no significant heterogeneity between the studies. The decision, which model must be used, the Fixed or the Random effects model, depends on heterogeneity. The chosen model is the Fixed effects model for MTHFR A1298C (ORG = 0.9849; 95%CI: 0.883-1.098) because the heterogeneity is not significant.

The 95% CI includes the value "1", as a result the ORG is not considered significant. So according to the Meta-analysis of GAS, it is concluded that, with the present data, the MTHFR (rs 1801131) polymorphism is not associated with CLL/SLL disease.

The number of studies was very limited, and only one study used Mixed population. All of the rest used Caucasians. As a result it is not possible to perform subgroup analysis for different ethnicities.

Table 3.4.2: Recessive model

Study	Patients CLL/SLL		Control		OR	95% CI
	CC	Total	CC	Total		
Gra et al. 2007	6	83	14	177	0.91	(0.34 -2.45)
Rudd et al. 2004	72	832	85	886	0.89	(0.64 -1.24)
Lim et al. 2006	104	1124	81	935	1.07	(0.79 -1.46)
Nuckel et al. 2004	12	111	7	102	1.65	(0.62 -1.36)

The forest plot of GAS (based on the recessive model) is presented below:

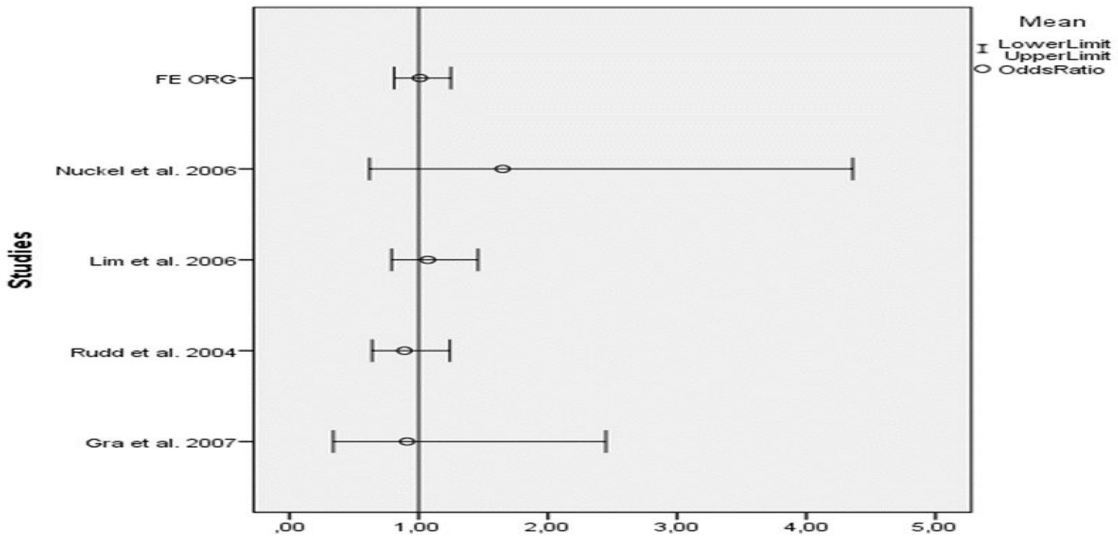


Figure 1. The forest plot of GAS. The x-axis is OR (95% CI) and y-axis are the studies.

Publication bias

The funnel plot is a simple scatter plot, which is able to identify publication bias in meta-analysis, but it does not provide an extended prediction (Lau et al. 2006). In our case it is not easy to determine whether the funnel plot is asymmetric, which would indicate publication bias. The variable depicted in the y-axis is the precision (1/SE) (Lee.2015).

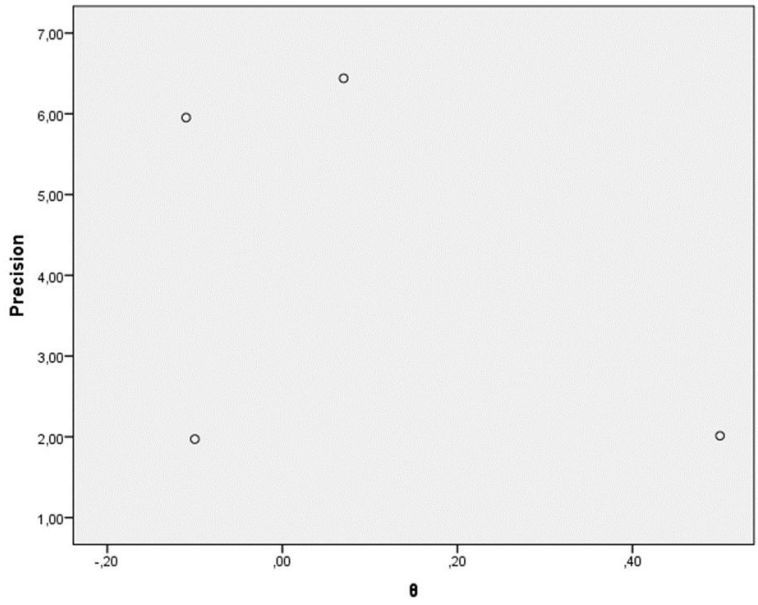


Figure 2. The funnel plot of GAS. The x-axis is theta (Θ) and y-axis is Precision (1/SE)

In the present study Egger's test was used (linear regression). If the p-value is greater than 0.05, it is not possible to reject the null hypothesis, which is that: there is not publication Bias. The p-value is equal to 0.598, greater than 0.05, so the publication bias does not appear to be statistically significant (Egger et al. 1997).

4. DISCUSSION

The purpose of the present study is to conduct a meta-analysis for the association of the MTHFR (rs1801131) with CLL/SLL, after having investigated the quality of reporting of genetic association studies for MTHFR (rs1801131) in chronic lymphocytic leukemia using the STREGA statement. In July 2015, a search in PubMed and HuGE databases was conducted. A total of 52 articles were found, with only 4 of them being relative to the subject. The limited number of eligible studies is due to the fact that only few of them use patients with CLL/SLL and investigate the MTHFR polymorphism of interest (rs1801131).

The results of evaluation of quality reporting, based on STREGA statement, from highest to lowest, were: 17.04/22 for Lim et al. 2006, 14.41/22 for Nuckel et al. 2004, 11.35/22 for Rudd et al. 2004 and for Gra et al.2007, 7.76/22.

The results of quality reporting are related to the impact factor of the journal, where the article was published. Lim et al. 2006 had the greatest score (17.04) with the impact factor of BLOOD, in which it was published, being 10.452 (2014). Nuckel et al. 2004 scored (14.41/22) with the impact factor of LEUKEMIA being 10.431(2014), Rudd et al. 2004 scored 11.35/22 with the impact factor of the CANCER EPIDEMIOLOGY, BIOMARKERS & PREVENTION being 4.125 and finally, Gra et al. 2007 had a score of 7.76/22 with the impact factor of AMERICAN GOURNAL OF HEMATOLOGY being 3.798. Consequently, it is evident that there is a remarkable relation between the score each article receives from the STREGA and the Impact factor of the journal, in which the article is published. However it must be noted that the impact factors stated above are for the year 2014 and the articles were published from 2004 to 2007. The most recent article, which was published in 2007 had the lowest score, while the article with the highest score was published in 2006. Thus the publication year does not appear to be related to the quality reporting.

Overall, only one of articles reported the study design in the title or abstract and used the usual term to describe it. This is a major methodological gap, because it is not easy to quickly identify the type of the study. It is very important to report that all of the studies were case – controls. Moreover, there was not any explanation on how they calculated the sample size.

In addition Little et al. 2009, stated that there is a distinction between laboratories (high and lower throughput) causing them to have different error rates. However, not on a single article was there any information about the state of laboratory, that could affect the results and influence the error rate.

Furthermore, no information was given about whether the articles' data were available at another source. Only Lim et al. 2006 mentions that there were missing data. All the other articles did not

provide information neither about the missing data nor about whether all samples were genotyped successfully. Also, only Lim et al. 2009 recorded information about a potential bias.

In the meta-analysis, the MTHFR (rs1801131) polymorphism was not significantly associated with the risk for CLL/SLL, which is consistent with results by Zintzaras et al. 2009. It was not possible to conduct subgroup analysis by race, because the number of the studies was limited. The inadequate number of studies made it impossible to use the Harbord test to identify the effect of study size. Furthermore, it was not possible to use solely the high-quality studies in the Meta-analysis, due to that same limitation. Publication bias was not significant, in the relevant articles.

The limitations of this study should be addressed. There was only a very limited number of studies that were eligible for our evaluation. Moreover, all of the articles were published before 2009, which is the year the STREGA statement became available. This is the most probable reason, why only a few of them cover the items of the STREGA checklist. Another limitation is that we confined our search to only two databases and a third database (e.g. EMBASE) was not used.

Regarding the scoring, it could also slightly vary among different evaluators, depending on their personal point of view. This process could introduce a potential bias, which could be restricted if the articles were also assessed by a second evaluator.

The STREGA statement, was only used by Ghazali et al. 2012 and Terrazzino et al. 2013 as a replacement of PICO for meta-analysis and Data Extraction. No articles evaluating the reporting quality of studies with the same or related subject to this study, were found. It should be emphasized that there is no available information, about a standard threshold that dictates that a study is of poor or good quality. Therefore, for our evaluation, it was necessary to compare and contrast the four articles with each other.

In conclusion, genetic articles appear to have methodological errors that could be avoided with the use of the recommendations given in the STREGA statement. The necessity of a document that elaborates on the STREGA recommendations, same as the STROBE Explanation and Elaboration document (Vandenbroucke et al. 2007), is undeniable. Only after the improvement of quality and quantity of publications, there will be clear evidence of the possible genetic associations with CLL.

5. REFFERENCES

Cheson BD, Bennett JM, Grever M, Kay N, Keating MJ, O'Brien S et al. (1996) National Cancer Institute-sponsored Working Group guidelines for chronic lymphocytic leukemia: revised guidelines for diagnosis and treatment. *Blood 1996;* ***87****: 4990–4997.*

Gemmati D, Ongaro A, Tognazzo S, Catozzi L, Federici F., Mauro E., Porta M. D, Campioni D, Bardi A., Gilli, Pellati A, Caruso A, Scapoli G. L., De Mattei M. (2007) Methylenetetrahydrofolate Reductase C677T And A1298C Gene Variants In Adult Non-Hodgkin's Lymphoma Patients: Association With Toxicity And SURVIVAL *Haematologica April 2007* ***92****: 478-485; Doi:10.3324/haematol.10587*

Egger M, Davey Smith G, Schneider M, Minder C. (1997) Bias in meta-analysis detected by a simple, graphical test. *BMJ 1997;****315****:629-34*

Ghazali N., Shaw R. J. , Rogers N, S, Risk J. M. (2012) Genomic determinants of normal tissue toxicity after radiotherapy for head and neck malignancy: A systematic review Oral Oncology **48** (2012) 1090–1100

Goyette P, Sumner JS, Milos R, et al. (1994) Human methylenetetrahydrofolate reductase: isolation of cDNA mapping and mutation identification. *Nat Genet 1994;****7****:551*

Goyette P., Pai A, Milos R, Frosst P, Tran P, Chen Z, Chan M, Rozen R. (1999) Gene structure of human and mouse methylenetetrahydrofolate reductase (MTHFR). *Mamm Genome 1999 Feb;****10****(2):204.*

Gra OA, Glotov AS, Nikitin EA, et al. Polymorphisms in xenobiotic-metabolizing genes and the risk of chronic lymphocytic leukemia and non-Hodgkin's lymphoma in adult Russian patients. *Am J Hematol. 2008;83(****4****):279–287*.

Huston T. J an d Cooper D. N. (2009) STREGA: a 'How-To' guide for reporting genetic associations *Hum Genet (2009)* ***125****:117–118*

He J, Liao X. Y., Zhu H. j,, Xue W. O., Shen G. P., Huang S. Y, Chen W, Jia W. H. (2014) Association of MTHFR C677T and A1298C polymorphisms withnon-Hodgkin lymphoma susceptibility: Evidence from a meta-analysis *SCIENTIFIC REPORTS | **4** : 6159 | . DOI: 10.1038/srep06159*

Kalila N. and Chesonb B. D. (1999) Chronic Lymphocytic Leukemia *The Oncologist 1999;****4****:352-369*

Lau j., Ioannidis J, P, A, Terrin N., Schmid C. H., Olkin I (2006) The case of the misleading funnel plot *MJ 2006;****333****:597–600*

Lee Y. H., (2015) Meta-Analysis of Genetic Association Studies Ann Lab Med 2015;**35**:283-287

Little J, Higgins J.,, Ioannidis P. H. G, et al (2009) STrengthening the REporting of Genetic Association studies (STREGA) – an extension of the STROBE statement *European Journal of Clinical Investigation Vol* ***39****. 247-266*

Lim U, Wang SS, Hartge P, et al. Gene-nutrient interactions among determinants of folate and one-carbon metabolism on the risk of non-Hodgkin lymphoma: NCI-SEER case-control study. Blood. 2007;109(**7**):3050–3059.

Nuckel H, Frey UH, Du¨rig J, et al. Methylenetetrahydrofolate reductase (MTHFR) gene 677C>T and 1298A>C polymorphisms are associated with differential apoptosis of leukemic B cells in vitro and disease progression in chronic lymphocytic leukemia. *Leukemia. 2004;18(****11****):1816–1823.*

Robien K, Ulrich CM. (2003) 5,10-Methylenetetrahydrofolate reductase polymorphisms and leukemia risk: a HuGE minireview. *Am JμEpidemiol 2003;**157**:571 – 82*.

Rozman C. and Montserrat E. (1995) Chronic Lymphocytic Leukemia *N Engl J MED 1995; **333**:1052-1057*

Rudd MF, Sellick GS, Allinson R, et al. MTHFR polymorphisms and risk of chronic lymphocytic leukemia. *Cancer Epidemiol Biomarkers Prev. 2004;13(**12**):2268–2270.*

Semenza JC, Delfino RJ, Ziogas A, Anton-Culver H. (2003) Breast cancer risk and methylenetetrahydrofolate reductase polymorphism. *Breast Cancer Res Treat 2003; **77**: 217–223.*

Juo H. H. and Ruey-Tay Lin (2013) Lack of association between a functional variant of the BRCA-1 related associated protein (BRAP) gene and ischemic stroke *BMC Medical Genetics 2013, 14:17*

Terrazzino S, Cargnin S, Del Re M, Danesi R, CanonicoP. GenazzaniA.A, (2013) DPYD IVS14+1G>A and 2846A>T genotyping for the prediction of severe fluoropyrimidine-related toxicity: a meta-analysis *Pharmacogenomics (2013) 14(**11**), 1255–1272*

Skibola CF, Smith MT, Kane E, Roman E, Rollinson S, Cartwright RA et al. (1999) Polymorphisms in the methylenetetrahydrofolate reductase gene are associated with susceptibility to acute leukemia in adults. *Proc Natl Acad Sci USA 1999; **96**: 12810–12815.*

Sun j., Hu j, Tu C., Zhong A., Xu H (2015) Obstructive Sleep Apnea Susceptibility Genes Chinese Population: A Field Synopsis and Meta-Analysis of Genetic Association Studies. *PLoS ONE 10(**8**): e0135942. doi:10.1371/journal.pone.0135942*

Vandenbroucke JP, von Elm E, Altman DG, Gotzsche PC, Mulrow CD, Pocock SJ, et al. (2007) Strengthening the Reporting of Observational Studies in Epidemiology (STROBE): explanation and elaboration. *PLoS Med. 2007;**4:**e297*

Wang SS, Davis S, Cerhan JR, et al. Polymorphisms in oxidative stress genes and risk for non-Hodgkin lymphoma. *Carcinogenesis. 2006;**27**: 1828-1834*.

Zintzaras E. and Kitsios D. G. (2009) Synopsis and Synthesis of Candidate-Gene Association Studies in Chronic Lymphocytic Leukemia: The CUMAGAS-CLL Information System *Am J Epidemiol 2009;**170**:671–678*

Zintzaras E (2010): The generalized odds ratio as a measure of genetic risk effect in the analysis and meta-analysis of association studies. *Stat Appl Genet Mol Biol 2010, **9**:Article21*

6. APPENDICES

6.1 Title: MTHFR Polymorphisms and Risk of Chronic Lymphocytic Leukemia

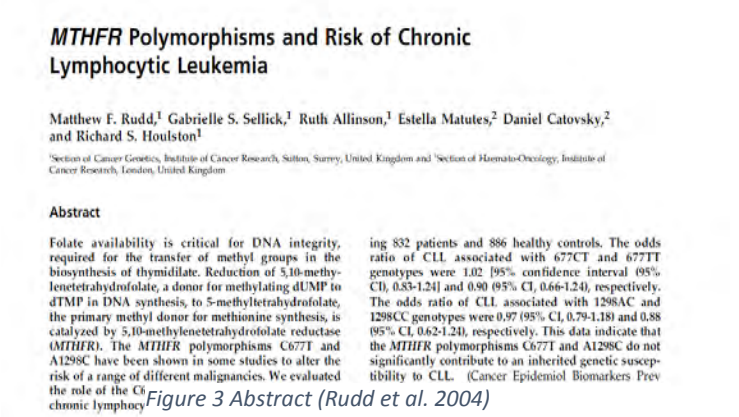
Authors: Matthew F. Rudd, Gabrielle S. Sellick, Ruth Allinson, Estella Matutes, Daniel Catovsky and Richard S. Houlston.

Cancer Epidemiol Biomarkers Prev 2004; 13(12).

6.1.1 Title and Abstract

Item 1a: There is not the usual term in the title or the abstract.

Item 1b: There is a balanced summary because is not separate the background, design, patients, measurements, results, limitations and conclusions.



***MTHFR* Polymorphisms and Risk of Chronic Lymphocytic Leukemia**

Matthew F. Rudd,[1] Gabrielle S. Sellick,[1] Ruth Allinson,[1] Estella Matutes,[2] Daniel Catovsky,[2] and Richard S. Houlston[1]

[1]Section of Cancer Genetics, Institute of Cancer Research, Sutton, Surrey, United Kingdom and [2]Section of Haemato-Oncology, Institute of Cancer Research, London, United Kingdom

Abstract

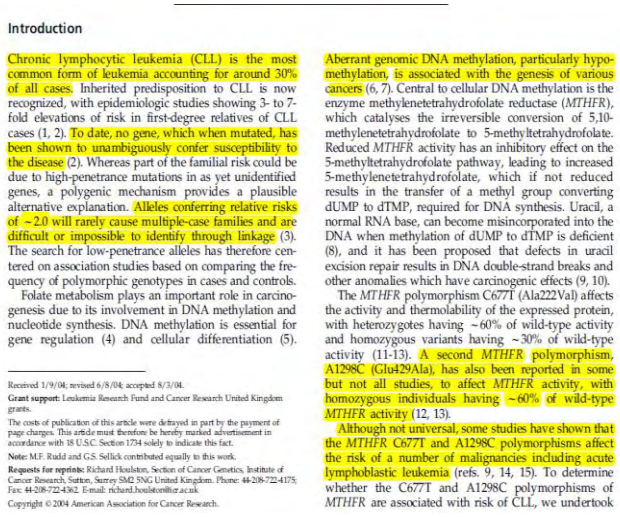
Folate availability is critical for DNA integrity, required for the transfer of methyl groups in the biosynthesis of thymidilate. Reduction of 5,10-methylenetetrahydrofolate, a donor for methylating dUMP to dTMP in DNA synthesis, to 5-methyltetrahydrofolate, the primary methyl donor for methionine synthesis, is catalyzed by 5,10-methylenetetrahydrofolate reductase (*MTHFR*). The *MTHFR* polymorphisms C677T and A1298C have been shown in some studies to alter the risk of a range of different malignancies. We evaluated the role of the C6 chronic lymphocy ing 832 patients and 886 healthy controls. The odds ratio of CLL associated with 677CT and 677TT genotypes were 1.02 [95% confidence interval (95% CI), 0.83-1.24] and 0.90 (95% CI, 0.66-1.24), respectively. The odds ratio of CLL associated with 1298AC and 1298CC genotypes were 0.97 (95% CI, 0.79-1.18) and 0.88 (95% CI, 0.62-1.24), respectively. This data indicate that the *MTHFR* polymorphisms C677T and A1298C do not significantly contribute to an inherited genetic susceptibility to CLL. (Cancer Epidemiol Biomarkers Prev

Figure 3 Abstract (Rudd et al. 2004)

6.1.2 Introduction

Item 2: There is scientific background of the study that provides important context for readers. (Vandenbroucke et al., 2007)

Item 3: There is not any information if this is the first report of a genetic association in CLL. There is only one important key-point, that the MTHFR affects the risk of a number of malignancies including ALL.



Introduction

Chronic lymphocytic leukemia (CLL) is the most common form of leukemia accounting for around 30% of all cases. Inherited predisposition to CLL is now recognized, with epidemiologic studies showing 3- to 7-fold elevations of risk in first-degree relatives of CLL cases (1, 2). To date, no gene, which when mutated, has been shown to unambiguously confer susceptibility to the disease (2). Whereas part of the familial risk could be due to high-penetrance mutations in as yet unidentified genes, a polygenic mechanism provides a plausible alternative explanation. Alleles conferring relative risks of ~2.0 will rarely cause multiple-case families and are difficult or impossible to identify through linkage (3). The search for low-penetrance alleles has therefore centered on association studies based on comparing the frequency of polymorphic genotypes in cases and controls.

Folate metabolism plays an important role in carcinogenesis due to its involvement in DNA methylation and nucleotide synthesis. DNA methylation is essential for gene regulation (4) and cellular differentiation (5). Aberrant genomic DNA methylation, particularly hypomethylation, is associated with the genesis of various cancers (6, 7). Central to cellular DNA methylation is the enzyme methylenetetrahydrofolate reductase (*MTHFR*), which catalyses the irreversible conversion of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate. Reduced *MTHFR* activity has an inhibitory effect on the 5-methyltetrahydrofolate pathway, leading to increased 5-methylenetetrahydrofolate, which if not reduced results in the transfer of a methyl group converting dUMP to dTMP, required for DNA synthesis. Uracil, a normal RNA base, can become misincorporated into the DNA when methylation of dUMP to dTMP is deficient (8), and it has been proposed that defects in uracil excision repair results in DNA double-strand breaks and other anomalies which have carcinogenic effects (9, 10).

The *MTHFR* polymorphism C677T (Ala222Val) affects the activity and thermolability of the expressed protein, with heterozygotes having ~60% of wild-type activity and homozygous variants having ~30% of wild-type activity (11-13). A second *MTHFR* polymorphism, A1298C (Glu429Ala), has also been reported in some but not all studies, to affect *MTHFR* activity, with homozygous individuals having ~60% of wild-type *MTHFR* activity (12, 13).

Although not universal, some studies have shown that the *MTHFR* C677T and A1298C polymorphisms affect the risk of a number of malignancies including acute lymphoblastic leukemia (refs. 9, 14, 15). To determine whether the C677T and A1298C polymorphisms of *MTHFR* are associated with risk of CLL, we undertook

Received 1/9/04; revised 6/8/04; accepted 8/3/04.
Grant support: Leukemia Research Fund and Cancer Research United Kingdom grants.
The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
Note: M.F. Rudd and G.S. Sellick contributed equally to this work.
Requests for reprints: Richard Houlston, Section of Cancer Genetics, Institute of Cancer Research, Sutton, Surrey SM2 5NG United Kingdom. Phone: 44-208-722-4175; Fax: 44-208-722-4362. E-mail: richard.houlston@icr.ac.uk

Figure 4 Introduction (Rudd et al. 2004)

6.1.3 Methods

Item 4: Case-control study (in the end of introduction)

Item 5: United Kingdom, Royal Marsden Hospital NHS and National Cancer Research Network Trial 1999-2002 and 1999-2002.

Controls were the spouses of patients with nonhematologic malignancies and were selected to closely match the age and sex of the cases. None of the controls had a personal history of CLL or other malignancy.

Item 6a: There is not any information on the criteria and methods for selection of subsets of participants from a larger study. Patients with nonhematologic malignancies and were selected to closely match the age and sex of the cases

Item 7a: To determine whether the C677T and A1298C polymorphisms of MTHFR are associated with risk of CLL.

Item 7b: Clearly define the genetic variant and to define the population stratification have used the distribution of genotypes in controls was tested for a departure from Hardy-Weinberg equilibrium. Also all the patients are British Caucasians.

Item 8a: Controls selected to be match the age and sex of the cases. The method was used to detection MTHFR genotype use described.

Item 8b: Describe the laboratory method (PCR), include source and storage of DNA, genotyping method and platform. There is no information about error rates, call rates, state of laboratory.

Materials and Methods

Patients. Eight hundred and thirty-two patients with adult CLL (577 males, 255 females; mean age at diagnosis 61 years; range 21-94; SD ± 12) referred to the Royal Marsden Hospital NHS Trust were studied. One hundred and nineteen of the cases (76 males, 43 females; mean age at diagnosis 59 years; range 26-93; SD ± 12) had one or more first-degree relatives affected with CLL. Eight hundred and eighty-six healthy individuals were recruited as part of either the National Cancer Research Network Trial (1999-2002), the Royal Marsden Hospital Trust/Institute of Cancer Research Family History and DNA Registry (1999-2004), or the National Study of Colorectal Cancer Genetics Trial (2004), all established within the United Kingdom. Controls (523 males, 363 females; mean age 60 years; range 21-91; SD ± 12) were the spouses of patients with nonhematologic malignancies and were selected to closely match the age and sex of the cases. None of the controls had a personal history of CLL or other malignancy. All cases and controls were British Caucasians, and there were no obvious differences in the demography of cases and controls in terms of place of residence within the United Kingdom. Samples were obtained with informed consent and ethical review board approval in accordance with the tenets of the Declaration of Helsinki.

Detection of *MTHFR* C677T and A1298C Genotypes. *MTHFR* C677T and A1298C genotypes were generated using Taqman technology implemented on an Applied Biosystems 7900HT sequence detection system. PCR reactions for detection of the C677T polymorphism contained 6.25 μL ABI Taqman PCR Master Mix, 0.16 μL ABI SNP assay-by-design master mix containing 900 mol/L forward primer (5′-gCACTTgAAggAgAAgg-TgTCT-3′), 900 nmol/L reverse primer (5′-CCTCAAA-gAAAAgCTgCgTgATg-3′), 200 nmol/L VIC-labeled MGB probe (5′-gCgggAgCCgATTTCAT[NFQ]-3′) and 200 nmol/L FAM-labeled MGB probe (5′-gCgggAgTC-gATTTCAT[NFQ]-3′), 4 μL (10 ng) of template DNA, and double-distilled water to a final volume of 12.5 μL. PCR reactions for detection of the A1298C polymorphism contained 6.25 μL ABI Taqman PCR Master Mix, 0.16 μL ABI SNP assay-by-design master mix containing 900 nmol/L of primers (5′-ggAggAgCTgCTgAAgATgTg-3′) and (5′-TggTTCTCCCgAgAggTAAAgA-3′), 200 nmol/L VIC-labeled MGB probe (5′-ACCAgTgAAgAAAg-TgT[NFQ]-3′) and 200 nmol/L FAM-labeled MGB probe (5′-CAgTgAAgCAAgTgT[NFQ]-3′), and DNA and water as above. All amplifications consisted of an initial denaturation at 95°C for 10 minutes followed by 40 cycles of denaturation (92°C/15 s) and extension (60°C/1 min).

Figure 5 Materials and Methods (Rudd et al. 2004)

Item 9a: Controls selected to be match the age and sex of the cases. No other information.

Item 9b: No information.

Item 10: Not information about study size

Item 11: No information about treatment.

Item 12a: STATA version 7.0 (Stata Corporation, College Station, TX) POWER version 1.30

Item 12b: logistic regression (adjusting for age and sex) X2 test

Item 12 c: Not information about missing data

Item 12d: Controls selected to be match the age and sex of the cases.

Item 12e: No information of any sensitivity analysis.

Item 12f: The controls were tested for HWE.

Item 12g: An unequal distribution of haplotype frequencies between cases and controls was tested for by means of the X^2test, where L1 is the likelihood of cases and controls analyzed together and L0 the sum of the individual likelihoods.

Item 12h: To test for population stratification, the distribution of genotypes in controls was tested for a departure from Hardy Weinberg equilibrium. The observed frequencies in the controls for both polymorphisms of MTHFR were in accordance with Hardy-Weinberg laws of equilibrium, providing no evidence of population stratification within the data set.

Item 12i: No information

Item 12j: No information

Statistical Analyses. The relationship between *MTHFR* genotypes and risk of CLL was assessed by means of the odds ratio (OR) with 95% confidence interval (95% CI) calculated using both conditional and unconditional logistic regression (adjusting for age and sex). Because both models yielded very similar results, only those for conditional logistic regression are presented. A test for trend (P_{trend}) in increasing CLL risk by having more than one putative high-risk allele or genotype was evaluated by means of the χ^2 test. To test for population stratification, the distribution of genotypes in controls was tested for a departure from Hardy-Weinberg equilibrium. An unequal distribution of haplotype frequencies between cases and controls was tested for by means of the χ^2 statistic $2(\ln L_1 - \ln L_0)$, where L_1 is the likelihood of cases and controls analyzed together and L_0 the sum of the individual likelihoods. All computations were undertaken using the statistical software package STATA version 7.0 (Stata Corporation, College Station, TX). Power calculations were undertaken using the method published by Fleiss et al. (17) as implemented in the statistical program POWER version 1.30 (Epicenter Software, http://icarus2.hsc.usc.edu/epicenter). $P = 0.05$ was considered statistically significant in all analyses.

Figure 6 Statistical Analysis (Rudd et al., 2004)

Results and Discussion

The observed frequencies in the controls for both polymorphisms of *MTHFR* were in accordance with Hardy-Weinberg laws of equilibrium, providing no evidence of population stratification within the data set. The frequency of the *MTHFR* C677T and A1298C polymorphisms in the general population has been recently reported in a meta-analysis of 16 studies providing data on 5,389 individuals (18). The frequencies of both polymorphisms detected in our study are in close agreement with this report. Specifically, the frequencies in our study (and in the meta-analysis) are CC/AA, 12% (15%); CC/AC 22% (22%); CC/CC, 10% (8.5%); CT/AA, 22% (22%); CT/AC, 22% (20%); CT/CC, 0% (0.25%); TT/AA, 12% (11%); TT/AC, 0% (0.46%); and TT/CC, 0% (0.03%). As was the case with the meta-analysis (18), no individuals with the *MTHFR* 677TT/1298AC, 677CT/1298CC, and 677TT/1298CC genotypes were identified.

Based on the number of cases and controls analyzed and the population frequencies of the rare alleles of each polymorphism, our study has 90% power to show an OR of 0.7 associated with each variant stipulating a $P = 0.05$.

Table 1 details the ORs of CLL and associated 95% CIs associated with each genotype. Adjustment for age and sex made no significant difference to findings hence only unadjusted ORs are presented. Individually, there is no evidence that either polymorphism affects the risk of CLL. Odds ratios for possession of the *MTHFR* 677T and 1298C alleles were 0.97 (95% CI, 0.84-1.12) and 0.95 (95% CI, 0.82-1.10), respectively. Restricting the analysis to familial cases provided, no evidence of a relationship between *MTHFR* polymorphisms and risk of CLL [ORs

Figure 7 Results and Discussion (Rudd et al., 2004)

6.1.4 Results

Item 13: Numbers of individuals in whom genotyping was successful: 832 cases and 886 controls. No information about missing data. Not information about no participation at each stage and flow diagram.

Item 14: The frequencies in our study (and in the meta-analysis) are CC/AA, 12% (15%); CC/AC 22% (22%); CC/CC, 10% (8.5%); CT/AA, 22% (22%); CT/AC, 22% (20%); CT/CC, 0% (0.25%); TT/ AA, 12%(11%); TT/AC, 0% (0.46%); and TT/CC, 0%(0.03%). No information about missing data.

Item 15: Table 1

Item 16a: Associated 95% CIs associated with each genotype. Adjustment for age and sex made no significant difference to findings hence only unadjusted ORs are presented95% confidence interval. No information about confounders.

Item 16b: No information

Item 16c: No information

Item 16d: Adjustment for age and sex made no significant difference to findings hence only unadjusted ORs are presented???(multy analysis)

Item 17a: Provide no statistically significant evidence that MTHFR plays a role in development of CLL, we cannot entirely exclude a small effect in risk of CLL associated with MTHFR variants. Moreover, as there is evidence of a gene-environment effect with respect to folate metabolism and cancer risk, it is possible that polymorphisms of MTHFR may mediate CLL risk in the context of a low-folate diet. The distribution of haplotypes was not significantly different between cases and controls

Item 17b: The results were summarized in a table.

Item 17c: No information

A1298C genotypes and chronic lymphocytic leukemia risk

	Cases (*n*)	Controls (*n*)	OR	95% CI
MTHFR C677T				
CC	361	383	1.0*	—
CT	381	397	1.02	(0.83-1.24)
TT	90	106	0.90	(0.66-1.24)
MTHFR A1298C				
AA	397	412	1.0*	—
AC	363	389	0.97	(0.79-1.18)
CC	72	85	0.88	(0.62-1.24)
MTHFR C677T/A1298C				
CC/AA	118	107	1.0*†‡	—
CC/AC	171	191	0.81†	(0.58-1.13)
CC/CC	72	85	0.77†	(0.51-1.16)
CT/AA	189	199	0.86†	(0.62-1.20)
CT/AC	192	198	0.88	(0.63-1.22)
TT/AA	90	106	0.77‡	(0.52-1.13)

NOTE: Unadjusted ORs and 95% CIs are shown for 832 cases and 886 controls; adjusting for age and sex was not significant. *MTHFR* 677/1298 genotypes CT/CC, TT/AC, and TT/CC were not observed in this study.
*Reference group.
†P_{trend} = 0.18; 1298 AA/AC/CC within 677CC.
‡P_{trend} = 0.18; 677 CC/CT/TT within 1298AA.

Figure 8 Associations of MTHFR and CLL (Rudd et. al., 2004)

(0.03%). As was the case with the meta-analysis (18), no individuals with the *MTHFR* 677TT/1298AC, 677CT/1298CC, and 677TT/1298CC genotypes were identified.

Based on the number of cases and controls analyzed and the population frequencies of the rare alleles of each polymorphism, our study has 90% power to show an OR of 0.7 associated with each variant stipulating a $P = 0.05$.

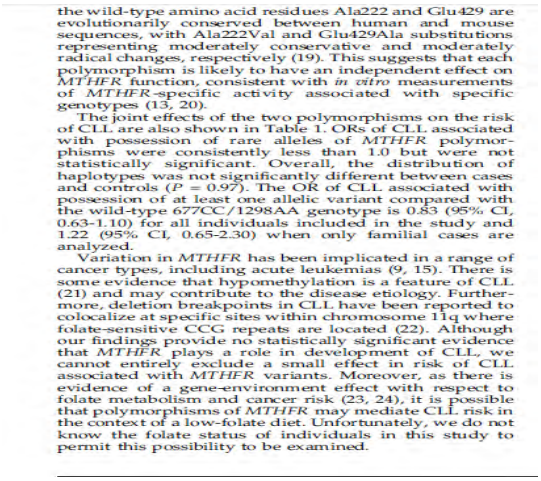
Table 1 details the ORs of CLL and associated 95% CIs associated with each genotype. Adjustment for age and sex made no significant difference to findings hence only unadjusted ORs are presented. Individually, there is no evidence that either polymorphism affects the risk of CLL. Odds ratios for possession of the *MTHFR* 677T and 1298C alleles were 0.97 (95% CI, 0.84-1.12) and 0.95 (95% CI, 0.82-1.10), respectively. Restricting the analysis to familial cases provided, no evidence of a relationship between *MTHFR* polymorphisms and risk of CLL [ORs for possession of 677T and 1298C alleles were 1.18 (95% CI, 0.89-1.56) and 0.87 (95% CI, 0.64-1.17), respectively].

There was evidence of significant linkage disequilibrium between the two polymorphisms ($P < 0.001$). In both cases and controls, we observed that subjects homozygous for either of the *MTHFR* mutations always possessed the wild-type genotype at the alternate site. For both *MTHFR* polymorphisms C677T and A1298C,

Figure 9 Results and Discussion No 2 (Rudd et al.,2004

6.1.5 Discussion

Item 18: Provide no statistically significant evidence that MTHFR plays a role in development of CLL, we cannot entirely exclude a small effect in risk of CLL associated with MTHFR variants. Moreover, as there is evidence of a gene-environment effect with respect to folate metabolism and cancer risk, it is possible that polymorphisms of MTHFR may mediate CLL risk in the context of a low-folate diet. The distribution of haplotypes was not significantly different between cases and controls.



the wild-type amino acid residues Ala222 and Glu429 are evolutionarily conserved between human and mouse sequences, with Ala222Val and Glu429Ala substitutions representing moderately conservative and moderately radical changes, respectively (19). This suggests that each polymorphism is likely to have an independent effect on *MTHFR* function, consistent with *in vitro* measurements of *MTHFR*-specific activity associated with specific genotypes (13, 20).

The joint effects of the two polymorphisms on the risk of CLL are also shown in Table 1. ORs of CLL associated with possession of rare alleles of *MTHFR* polymorphisms were consistently less than 1.0 but were not statistically significant. Overall, the distribution of haplotypes was not significantly different between cases and controls ($P = 0.97$). The OR of CLL associated with possession of at least one allelic variant compared with the wild-type 677CC/1298AA genotype is 0.83 (95% CI, 0.63-1.10) for all individuals included in the study and 1.22 (95% CI, 0.65-2.30) when only familial cases are analyzed.

Variation in *MTHFR* has been implicated in a range of cancer types, including acute leukemias (9, 15). There is some evidence that hypomethylation is a feature of CLL (21) and may contribute to the disease etiology. Furthermore, deletion breakpoints in CLL have been reported to colocalize at specific sites within chromosome 11q where folate-sensitive CCG repeats are located (22). Although our findings provide no statistically significant evidence that *MTHFR* plays a role in development of CLL, we cannot entirely exclude a small effect in risk of CLL associated with *MTHFR* variants. Moreover, as there is evidence of a gene-environment effect with respect to folate metabolism and cancer risk (23, 24), it is possible that polymorphisms of *MTHFR* may mediate CLL risk in the context of a low-folate diet. Unfortunately, we do not know the folate status of individuals in this study to permit this possibility to be examined.

Figure 10 Results and Discussion No 3 (Rudd et al., 2004)

Item 19: No information about a potential bias.

Item 20: Provide no statistically significant evidence that MTHFR plays a role in development of CLL, we cannot entirely exclude a small effect in risk of CLL associated with MTHFR variants. Moreover, as there is evidence of a gene-environment effect with respect to folate metabolism and cancer risk it is possible that polymorphisms of MTHFR may mediate CLL risk in the context of a low-folate diet. The frequency of the MTHFR C677T and A1298C polymorphisms in the general population has been recently reported in a meta-analysis of 16 studies providing data on 5,389 individuals. The frequencies of both polymorphisms detected in our study are in close agreement with this report.

Item 21: No information

Item 22: No information about conflicts of interest.

6.2. Title: Methylenetetrahydrofolate reductase (MTHFR) gene 677C4T and 1298A4C polymorphisms are associated with differential apoptosis of leukemic B cells in vitro and disease progression in chronic lymphocytic leukemia

Editors: H Nuckel, UH Frey, J Du¨ rig, U Du¨hrsen and W Siffer.

Methylenetetrahydrofolate reductase (MTHFR) regulates the metabolism of folate and methionine, essential components of DNA synthesis and methylation. We investigated whether the two genetic MTHFR polymorphisms (677C>T and 1298A>C) are associated with an increased risk for chronic lymphocytic leukemia (CLL) or may predict disease progression. Moreover, we measured potential genotype effects on apoptosis of B-CLL cells.

Allele frequencies and genotype distributions for both polymorphisms were not significantly different in 111 patients *vs* 92 healthy controls. While progression-free survival (PFS) was not significantly different in individuals with CLL including all stages, in patients with Binet stage A PFS was significantly longer in patients displaying the MTHFR 677CC ($P=0.043$) and the MTHFR 1298A/C or CC genotypes ($P=0.019$). In a multivariate analysis, MTHFR haplotype (677CC plus 1298CC or A/C) was the best independent prognostic factor for PFS compared with other known prognostic factors. Spontaneous apoptosis of B-CLL cells *in vitro* was significantly increased in the favorable risk group with MTHFR 677CC and MTHFR 1298AC, which may constitute the cellular basis of the observed associations. While MTHFR polymorphisms do not affect the risk for B-CLL, they may be independent prognostic markers that influence the PFS in patients with early-stage B-CLL.
Leukemia (2004) 18, 1816–1823. doi:10.1038/sj.leu.2403484
Published online 16 September 2004
Keywords: chronic lymphocytic leukemia; cancer; MTHFR; genetic polymorphism; apoptosis; folic acid

6.2.1 Title and Abstract

Item 1a: There is not the usual term in the title or the abstract

Item 1b: There is a balanced summary because is not separate the background, design, patients, measurements, results, limitations and conclusions.

Figure 11 Abstract (Nuckel et al., 2004)

6.2.2 Introduction

Item 2: There is scientific background of the study that provides important context for readers. (Vandenbroucke et al., 2007)

Item 3: There is not any information if this is the first report of a genetic association in CLL. There is only one important key-point, that the MTHFR affects the risk of a number of malignancies including ALL.

Introduction

B-cell chronic lymphocytic leukemia (B-CLL) is a heterogeneous disease with a highly variable clinical course. Staging systems devised by Rai[1] and Binet[2] are useful for predicting survival and treatment requirements in patients with CLL. However, these staging systems are of limited prognostic value in early stages of the disease (Binet A or Rai stages 0–II), which applies for most of the patients at first diagnosis. Therefore, a number of studies aimed at identifying novel prognostic markers, which may help define patient subgroups with favorable vs poor clinical outcome in early CLL.[3] The presence or absence of somatic mutations in the immunoglobulin heavy-chain variable region (IgVH) of B-CLL cells has been described as one of the most powerful prognostic factors, as B-CLL cases with mutated IgVH genes exhibit a favorable clinical course while B-CLL patients with unmutated Ig VH genes are characterized by a poor outcome in terms of reduced survival and responsiveness to chemotherapy.[4–8]

Recently, Rosenwald *et al*[9] demonstrated that expression analysis of a single gene, the protein tyrosine kinase ZAP-70, could correctly predict the IgVH mutation status in 93% of patients. We could confirm previous studies by showing that ZAP-70 expression is a novel prognostic marker in B-CLL. Furthermore, our results and those of others suggest that a combination of prognostic factors such as ZAP-70 plus CD38 expression status may help optimize currently available prognostic instruments for the risk stratification of CLL patients in clinical trials.[10]

A refined risk stratification of CLL patients, especially for those patients in stage A according to Binet, may in future guide more specific, tailored treatment decisions for an individual patient.

Common single-nucleotide polymorphisms (SNPs) in the 5,10-methylenetetrahydrofolate reductase (MTHFR) gene (677C>T and 1298A>C) have been associated with the risk for adult and childhood acute lymphocytic leukemia.[11–13]

The enzyme MTHFR catalyzes the reduction of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, which is the carbon donor for *de novo* methionine synthesis and DNA methylation. Approximately 10% of Caucasian populations are homozygous for a common C>T substitution at nucleotide 677 of the MTHFR gene. This mutation converts an alanine to a valine at codon 225 and is associated with reduced enzyme activity. The potential influence of MTHFR activity on DNA

Figure 12 introduction (Nuckel et al. 2004

6.2.3 Methods

Item 4: No use of normal term of study design.

Item 5: The period was August 2001 and October 2003. All the patients were Caucasian. There is not information about location.

Item 6a: Indications for treatment were based on standard criteria by Cheson (1996)

Item 6b: Age-matched controls, 111 CLL cases and 92 controls.

Item 7a: Retrospective study of 111 CLL patients designed to evaluate the potential of the MTHFR 677C4T and 1298A4C gene polymorphisms as a prognostic factor for the natural course of B-CLL in patients with Binet stage A. In addition, we report the results of a multivariate analysis of the genotype data with other important prognostic factors in B-CLL. We investigated which cellular phenotype might contribute to the observed association. To study the impact of prognostic factors for a first-line therapy, a multivariate Cox regression analysis was performed.

Item 7b: Genetic variant is clearly define. All the patients were Caucasian.

Item 8a: Following the manufacturer's instructions and MTHFR genotypes were determined by PCRRFLP as described by Semenza et al., 2003 and Skiloba et al., 1999.

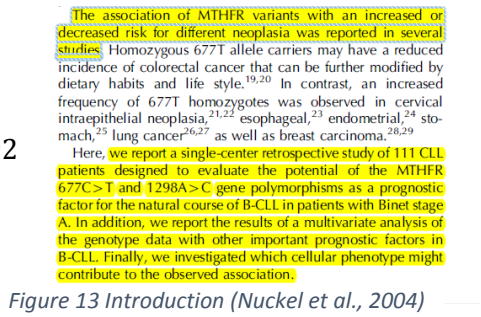
Item 8b: Describe the laboratory method (PCR), include source and storage of DNA, genotyping method and platform. There is not information about error rates, call rates, state of laboratory.

Item 9a: No other information.

Item 9b: No information about a potential bias.

Item 10: No information about study size.

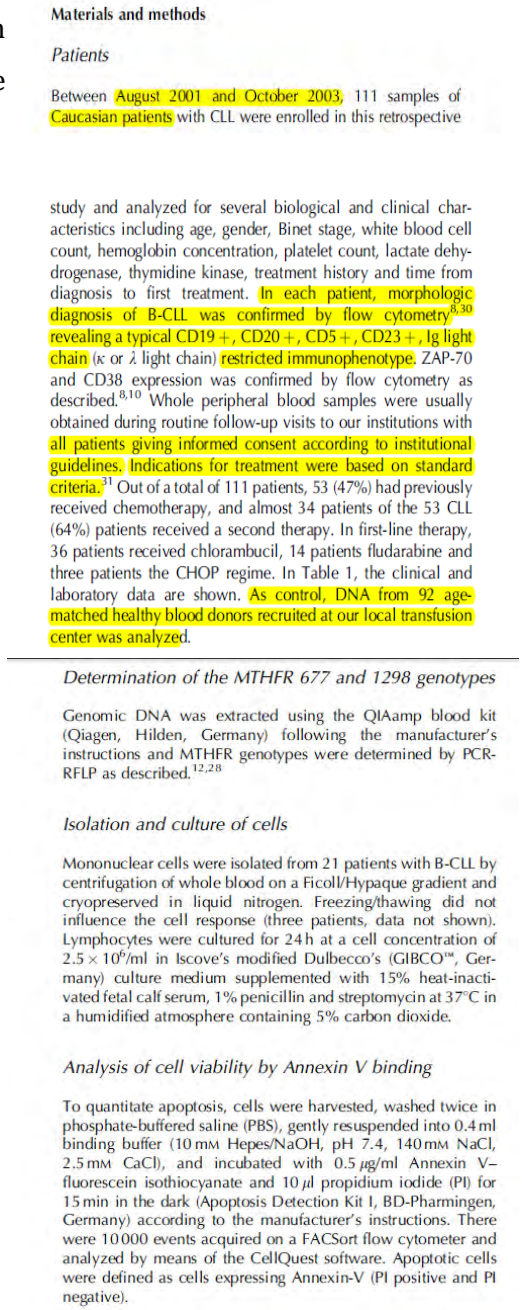
Item 11: Out of a total of 111 patients, 53 (47%) had previously received chemotherapy, and almost 34 patients of the 53 CLL (64%) patients received a second therapy. In first-line therapy, 36 patients received chlorambucil, 14 patients fludarabine and three patients the CHOP regime.



The association of MTHFR variants with an increased or decreased risk for different neoplasia was reported in several studies. Homozygous 677T allele carriers may have a reduced incidence of colorectal cancer that can be further modified by dietary habits and life style.[19,20] In contrast, an increased frequency of 677T homozygotes was observed in cervical intraepithelial neoplasia,[21,22] esophageal,[23] endometrial,[24] stomach,[25] lung cancer[26,27] as well as breast carcinoma.[28,29]

Here, we report a single-center retrospective study of 111 CLL patients designed to evaluate the potential of the MTHFR 677C>T and 1298A>C gene polymorphisms as a prognostic factor for the natural course of B-CLL in patients with Binet stage A. In addition, we report the results of a multivariate analysis of the genotype data with other important prognostic factors in B-CLL. Finally, we investigated which cellular phenotype might contribute to the observed association.

Figure 13 Introduction (Nuckel et al., 2004)



Materials and methods

Patients

Between August 2001 and October 2003, 111 samples of Caucasian patients with CLL were enrolled in this retrospective study and analyzed for several biological and clinical characteristics including age, gender, Binet stage, white blood cell count, hemoglobin concentration, platelet count, lactate dehydrogenase, thymidine kinase, treatment history and time from diagnosis to first treatment. In each patient, morphologic diagnosis of B-CLL was confirmed by flow cytometry[8,30] revealing a typical CD19+, CD20+, CD5+, CD23+, Ig light chain (κ or λ light chain) restricted immunophenotype. ZAP-70 and CD38 expression was confirmed by flow cytometry as described.[8,10] Whole peripheral blood samples were usually obtained during routine follow-up visits to our institutions with all patients giving informed consent according to institutional guidelines. Indications for treatment were based on standard criteria.[31] Out of a total of 111 patients, 53 (47%) had previously received chemotherapy, and almost 34 patients of the 53 CLL (64%) patients received a second therapy. In first-line therapy, 36 patients received chlorambucil, 14 patients fludarabine and three patients the CHOP regime. In Table 1, the clinical and laboratory data are shown. As control, DNA from 92 age-matched healthy blood donors recruited at our local transfusion center was analyzed.

Determination of the MTHFR 677 and 1298 genotypes

Genomic DNA was extracted using the QIAamp blood kit (Qiagen, Hilden, Germany) following the manufacturer's instructions and MTHFR genotypes were determined by PCR-RFLP as described.[12,28]

Isolation and culture of cells

Mononuclear cells were isolated from 21 patients with B-CLL by centrifugation of whole blood on a Ficoll/Hypaque gradient and cryopreserved in liquid nitrogen. Freezing/thawing did not influence the cell response (three patients, data not shown). Lymphocytes were cultured for 24 h at a cell concentration of 2.5×10^6/ml in Iscove's modified Dulbecco's (GIBCO™, Germany) culture medium supplemented with 15% heat-inactivated fetal calf serum, 1% penicillin and streptomycin at 37°C in a humidified atmosphere containing 5% carbon dioxide.

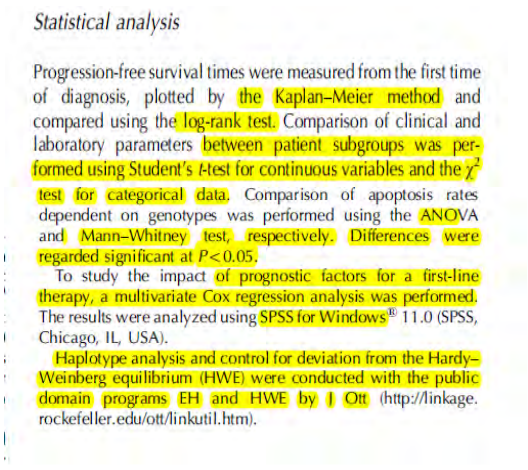
Analysis of cell viability by Annexin V binding

To quantitate apoptosis, cells were harvested, washed twice in phosphate-buffered saline (PBS), gently resuspended into 0.4 ml binding buffer (10 mM Hepes/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM CaCl), and incubated with 0.5 μg/ml Annexin V–fluorescein isothiocyanate and 10 μl propidium iodide (PI) for 15 min in the dark (Apoptosis Detection Kit I, BD-Pharmingen, Germany) according to the manufacturer's instructions. There were 10 000 events acquired on a FACSort flow cytometer and analyzed by means of the CellQuest software. Apoptotic cells were defined as cells expressing Annexin-V (PI positive and PI negative).

Figure 14 Materials and Methods (Nuckel et al. 2004)

Item 12a: SPSS 11.0 and Ott (http://linkage.rockefeller.edu/ott/linkutil.htm)

Item 12b: Kaplan–Meier method and compared using the log-rank test. Subgroups was performed using Student's t-test for continuous variables and the X^2 test for categorical data. Comparison of apoptosis rates dependent on genotypes was performed using the ANOVA and Mann–Whitney test, respectively. Differences were regarded significant at P=0.05.To study the impact of prognostic factors for a first-line\therapy, a multivariate Cox regression analysis.



Statistical analysis

Progression-free survival times were measured from the first time of diagnosis, plotted by the Kaplan–Meier method and compared using the log-rank test. Comparison of clinical and laboratory parameters between patient subgroups was performed using Student's *t*-test for continuous variables and the χ^2 test for categorical data. Comparison of apoptosis rates dependent on genotypes was performed using the ANOVA and Mann–Whitney test, respectively. Differences were regarded significant at $P<0.05$.

To study the impact of prognostic factors for a first-line therapy, a multivariate Cox regression analysis was performed. The results were analyzed using SPSS for Windows® 11.0 (SPSS, Chicago, IL, USA).

Haplotype analysis and control for deviation from the Hardy–Weinberg equilibrium (HWE) were conducted with the public domain programs EH and HWE by J Ott (http://linkage.rockefeller.edu/ott/linkutil.htm).

Figure 15 Statistical Analysis (Nuckel et al.2004)

Item 12 c: Not information about missing data.

Item 12d: Age-matched controls.

Item 12e: No information of any sensitivity analysis.

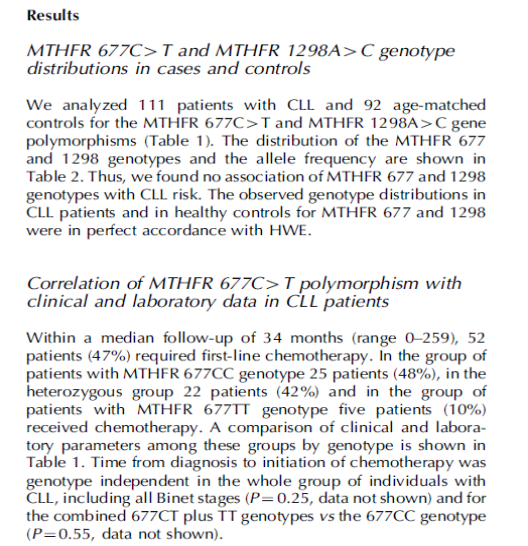
Item 12f: The controls were tested for HWE.

Item 12g: Haplotype analysis and control for deviation from the Hardy–Weinberg equilibrium (HWE) were conducted with the public domain programs EH and HWE by J Ott

Item 12h: Not information about stratification.

Item 12i: The impact of prognostic factors for a first-line therapy, a multivariate Cox regression analysis was performed.

Item 12j: No information



Results

MTHFR 677C>T and MTHFR 1298A>C genotype distributions in cases and controls

We analyzed 111 patients with CLL and 92 age-matched controls for the MTHFR 677C>T and MTHFR 1298A>C gene polymorphisms (Table 1). The distribution of the MTHFR 677 and 1298 genotypes and the allele frequency are shown in Table 2. Thus, we found no association of MTHFR 677 and 1298 genotypes with CLL risk. The observed genotype distributions in CLL patients and in healthy controls for MTHFR 677 and 1298 were in perfect accordance with HWE.

Correlation of MTHFR 677C>T polymorphism with clinical and laboratory data in CLL patients

Within a median follow-up of 34 months (range 0–259), 52 patients (47%) required first-line chemotherapy. In the group of patients with MTHFR 677CC genotype 25 patients (48%), in the heterozygous group 22 patients (42%) and in the group of patients with MTHFR 677TT genotype five patients (10%) received chemotherapy. A comparison of clinical and laboratory parameters among these groups by genotype is shown in Table 1. Time from diagnosis to initiation of chemotherapy was genotype independent in the whole group of individuals with CLL, including all Binet stages ($P=0.25$, data not shown) and for the combined 677CT plus TT genotypes *vs* the 677CC genotype ($P=0.55$, data not shown).

Figure 16 Results (Nuckel et al., 2004)

6.2.4 Results

Item 13: Analyzed 111 patients with CLL and 92 age-matched controls for the MTHFR 677C4T and MTHFR 1298A4C gene polymorphism. But no information about missing genotyping. No information about non-participation at each stage or a flow diagram.

Item 14: Table 1.

Item 15: Table 2.

Item 16a: 95% confidence interval. No information about confounders.

Item 16b: No information.

Item 16c: No information.

Item 16d: Multivariate (Cox) analysis for independent prognostic factors. Both MTHFR polymorphisms are independent factors if only adjusted for gender and age.

Item 17a: Correlation of MTHFR 1298A4C gene polymorphism with clinical and laboratory data in CLL patients. Haplotype analysis of MTHFR 677C4T and MTHFR. Multivariate (Cox) analysis for independent prognostic factors. Correlation of the MTHFR 677C4T and MTHFR1298A4C polymorphisms with clinical and laboratory data in ZAP-70-negative CLL 1298A4C gene polymorphism. Increased spontaneous apoptosis in B cells from CLL patients with the MTHFR 677CC and MTHFR1298AC/CC polymorphisms

Item 17b: The results were summarized in tables.

Item 17c: No information.

Table 1 Clinical and laboratory data at diagnosis in patients with MTHFR 677C>T and MT

Variable	*All patients*	*MTHFR 677CC genotype*	*MTHFR 677CT genotype*	*MT*
Number of patients	111	56	43	
Median age at diagnosis (years)	59	57	60.5	
Male	78 (70%)	40 (71%)	29 (67%)	
Binet stage ($N=102$)				
A	74 (73%)[a]	37 (73%)	29 (73%)	
B	22 (22%)	11 (22%)	9 (23%)	
C	6 (6%)	3 (6%)	2 (5%)	
Hemoglobin (g/dl) ($N=61$)	14.18±1.57[b]	14.38±1.13	13.82±2.07	14.
Platelets ($\times 10^{-3}/\mu l$) ($N=61$)	193±61	192±54	194±70	1
Leukocytes ($\times 10^{-3}/\mu l$) ($N=65$)	34.39±36.45	35.87±45.1	29.0±19.45	49.
CD38+leukemia ($N=104$)	37 (36%)	19 (38%)	14 (33%)	
ZAP-70+leukemia ($N=68$)	30 (44%)	14 (45%)	14 (47%)	
Thymidine kinase (IU/l) ($N=71$)	19.2±26.86	15.81±15.33	24.5±37.8	20.
β_2-Microglobulin (mg/l) ($N=19$)	2.15±0.52	2.03±0.53	2.22±0.44	2.

NS: nonsignificant.
[a]Percent of total number of patients.
[b]Mean±standard error of the mean.

Figure 17 Table (Nuckel et al. 2004

Table 2 Computed haplotypes and genotypes for the MTHFR 677C>T and 1298A>C polymorphisms in patients and controls

MTHFR 677 genotype	*MTHFR 1298 genotype*	*Number (%)*	*MTHFR 677 allele*	*MTHFR 1298 allele*	*Frequency*
Patients					
CC	AA	14 (13)	C	A	0.37
CT	AA	25 (23)	C	C	0.30
TT	AA	12 (11)	T	A	0.32
CC	AC	30 (27)	T	C	0.00
CT	AC	18 (16)			
CC	CC	12 (11)			
Controls					
CC	AA	13 (14)	C	A	0.36
CT	AA	21 (23)	C	C	0.33
TT	AA	11 (12)	T	A	0.29
CC	AC	23 (25)	T	C	0.00
CT	AC	17 (18)			
CC	CC	7 (8)			

Haplotypes were calculated using the data from 111 patients and 92 controls as described in 'Materials and methods'. Significant linkage between the indicated alleles was found for both cohorts ($P<0.001$; χ^2 test).

Figure 18 Table (Nuckel et al 2004)

6.2.5 Discussion

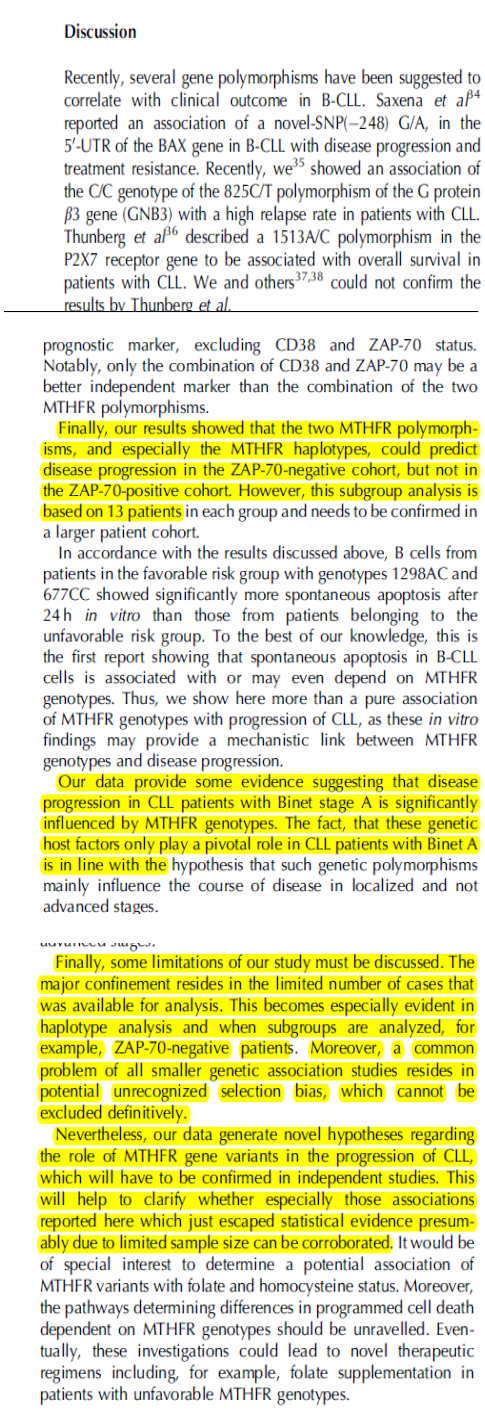
Item 18: Recently, it was suggested that polymorphisms at positions 677 and 1298 of the MTHFR gene are associated with reduced susceptibility for acute lymphocytic leukemia. Comparing frequencies of MTHFR genotypes in CLL patients' vs healthy controls, no significant differences were observed. Therefore, MTHFR polymorphisms are unlikely to influence the susceptibility to develop CLL. A strong linkage disequilibrium of these two polymorphisms was confirmed here.

Item 19: Finally, some limitations of our study must be discussed. The major confinement resides in the limited number of cases that was available for analysis. This becomes especially evident in haplotype analysis and when subgroups are analyzed, for example, ZAP-70-negative patients. Moreover, a common problem of all smaller genetic association studies resides in potential unrecognized selection bias, which cannot be excluded definitively.

Item 20: The data generate novel hypotheses regarding the role of MTHFR gene variants in the progression of CLL, which will have to be confirmed in independent studies. This will help to clarify whether especially those associations reported here which just escaped statistical evidence presumably due to limited sample size can be corroborated. It would be of special interest to determine a potential association of MTHFR variants with folate and homocysteine status. Some limitations of our study must be discussed. The major confinement resides in the limited number of cases that was available for analysis. This becomes especially evident in haplotype analysis and when subgroups are analyzed, for example, ZAP-70-negative patients. Moreover, a common problem of all smaller genetic association studies resides in potential unrecognized selection bias, which cannot be excluded definitively.

Item 21: The data generate novel hypotheses regarding the role of MTHFR gene variants in the progression of CLL, which will have to be confirmed in independent studies.

Item 22: Financial support by Essen Medical School IFORES program.



Discussion

Recently, several gene polymorphisms have been suggested to correlate with clinical outcome in B-CLL. Saxena *et al*[34] reported an association of a novel-SNP(−248) G/A, in the 5′-UTR of the BAX gene in B-CLL with disease progression and treatment resistance. Recently, we[35] showed an association of the C/C genotype of the 825C/T polymorphism of the G protein β3 gene (GNB3) with a high relapse rate in patients with CLL. Thunberg *et al*[36] described a 1513A/C polymorphism in the P2X7 receptor gene to be associated with overall survival in patients with CLL. We and others[37,38] could not confirm the results by Thunberg *et al*.

prognostic marker, excluding CD38 and ZAP-70 status. Notably, only the combination of CD38 and ZAP-70 may be a better independent marker than the combination of the two MTHFR polymorphisms.

Finally, our results showed that the two MTHFR polymorphisms, and especially the MTHFR haplotypes, could predict disease progression in the ZAP-70-negative cohort, but not in the ZAP-70-positive cohort. However, this subgroup analysis is based on 13 patients in each group and needs to be confirmed in a larger patient cohort.

In accordance with the results discussed above, B cells from patients in the favorable risk group with genotypes 1298AC and 677CC showed significantly more spontaneous apoptosis after 24 h *in vitro* than those from patients belonging to the unfavorable risk group. To the best of our knowledge, this is the first report showing that spontaneous apoptosis in B-CLL cells is associated with or may even depend on MTHFR genotypes. Thus, we show here more than a pure association of MTHFR genotypes with progression of CLL, as these *in vitro* findings may provide a mechanistic link between MTHFR genotypes and disease progression.

Our data provide some evidence suggesting that disease progression in CLL patients with Binet stage A is significantly influenced by MTHFR genotypes. The fact, that these genetic host factors only play a pivotal role in CLL patients with Binet A is in line with the hypothesis that such genetic polymorphisms mainly influence the course of disease in localized and not advanced stages.

Finally, some limitations of our study must be discussed. The major confinement resides in the limited number of cases that was available for analysis. This becomes especially evident in haplotype analysis and when subgroups are analyzed, for example, ZAP-70-negative patients. Moreover, a common problem of all smaller genetic association studies resides in potential unrecognized selection bias, which cannot be excluded definitively.

Nevertheless, our data generate novel hypotheses regarding the role of MTHFR gene variants in the progression of CLL, which will have to be confirmed in independent studies. This will help to clarify whether especially those associations reported here which just escaped statistical evidence presumably due to limited sample size can be corroborated. It would be of special interest to determine a potential association of MTHFR variants with folate and homocysteine status. Moreover, the pathways determining differences in programmed cell death dependent on MTHFR genotypes should be unravelled. Eventually, these investigations could lead to novel therapeutic regimens including, for example, folate supplementation in patients with unfavorable MTHFR genotypes.

Figure 19 Discussion (Nuckel et al. 2004)

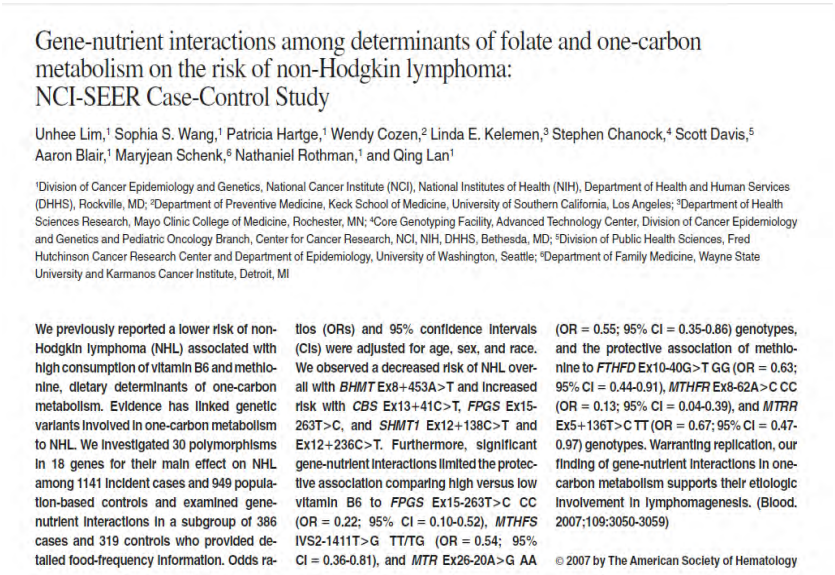
6.3 Title: Gene-nutrient interactions among determinants of folate and one-carbon metabolism on the risk of non-Hodgkin lymphoma: NCI-SEER Case-Control Study

Authors : Unhee Lim, Sophia S. Wang, Patricia Hartge, Wendy Cozen, Linda E. Kelemen, Stephen Chanock, Scott Davis, Aaron Blair, Maryjean Schenk, Nathaniel Rothman and Qing Lan

6.3.1 Title and Abstract

Item 1a: There is a usual term in the title or the abstract, Case – Control.

Item 1b: There is a balanced summary because is not separate the background, design, patients, measurements, results, limitations and conclusions. Also the investigator gives an overview of what is known on the topic and the gaps in current knowledge. (Vandenbroucke et al., 2007)



Gene-nutrient interactions among determinants of folate and one-carbon metabolism on the risk of non-Hodgkin lymphoma: NCI-SEER Case-Control Study

Unhee Lim,[1] Sophia S. Wang,[1] Patricia Hartge,[1] Wendy Cozen,[2] Linda E. Kelemen,[3] Stephen Chanock,[4] Scott Davis,[5] Aaron Blair,[1] Maryjean Schenk,[6] Nathaniel Rothman,[1] and Qing Lan[1]

[1]Division of Cancer Epidemiology and Genetics, National Cancer Institute (NCI), National Institutes of Health (NIH), Department of Health and Human Services (DHHS), Rockville, MD; [2]Department of Preventive Medicine, Keck School of Medicine, University of Southern California, Los Angeles; [3]Department of Health Sciences Research, Mayo Clinic College of Medicine, Rochester, MN; [4]Core Genotyping Facility, Advanced Technology Center, Division of Cancer Epidemiology and Genetics and Pediatric Oncology Branch, Center for Cancer Research, NCI, NIH, DHHS, Bethesda, MD; [5]Division of Public Health Sciences, Fred Hutchinson Cancer Research Center and Department of Epidemiology, University of Washington, Seattle; [6]Department of Family Medicine, Wayne State University and Karmanos Cancer Institute, Detroit, MI

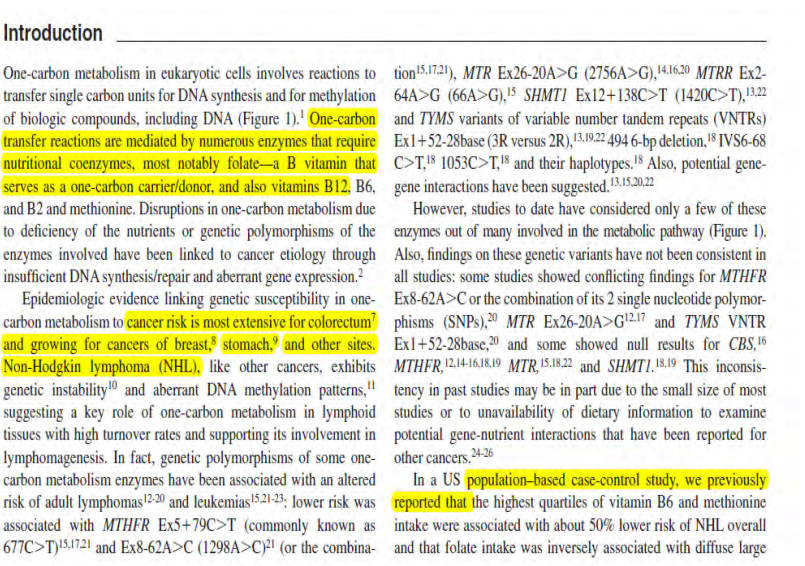
We previously reported a lower risk of non-Hodgkin lymphoma (NHL) associated with high consumption of vitamin B6 and methionine, dietary determinants of one-carbon metabolism. Evidence has linked genetic variants involved in one-carbon metabolism to NHL. We investigated 30 polymorphisms in 18 genes for their main effect on NHL among 1141 incident cases and 949 population-based controls and examined gene-nutrient interactions in a subgroup of 386 cases and 319 controls who provided detailed food-frequency information. Odds ratios (ORs) and 95% confidence intervals (CIs) were adjusted for age, sex, and race. We observed a decreased risk of NHL overall with *BHMT* Ex8+453A>T and increased risk with *CBS* Ex13+41C>T, *FPGS* Ex15-263T>C, and *SHMT1* Ex12+138C>T and Ex12+236C>T. Furthermore, significant gene-nutrient interactions limited the protective association comparing high versus low vitamin B6 to *FPGS* Ex15-263T>C CC (OR = 0.22; 95% CI = 0.10-0.52), *MTHFS* IVS2-1411T>G TT/TG (OR = 0.54; 95% CI = 0.36-0.81), and *MTR* Ex26-20A>G AA (OR = 0.55; 95% CI = 0.35-0.86) genotypes, and the protective association of methionine to *FTHFD* Ex10-40G>T GG (OR = 0.63; 95% CI = 0.44-0.91), *MTHFR* Ex8-62A>C CC (OR = 0.13; 95% CI = 0.04-0.39), and *MTRR* Ex5+136T>C TT (OR = 0.67; 95% CI = 0.47-0.97) genotypes. Warranting replication, our finding of gene-nutrient interactions in one-carbon metabolism supports their etiologic involvement in lymphomagenesis. (Blood. 2007;109:3050-3059)

Figure 20 Abstract 9lim et al. 2006)

6.3.2 Introduction

Item 2: There is scientific background of the study that provides important context for readers. (Vandenbroucke et al., 2007)

Item 3: Previously reported that the highest quartiles of vitamin B6 and methionine intake were associated with about 50% lower risk of NHL overall and that folate intake was inversely associated with diffuse large B-cell lymphoma (DLBCL).This is the first investigation of NHL that examined potential gene-nutrient interactions involving one-carbon metabolism.



Introduction

One-carbon metabolism in eukaryotic cells involves reactions to transfer single carbon units for DNA synthesis and for methylation of biologic compounds, including DNA (Figure 1).[1] One-carbon transfer reactions are mediated by numerous enzymes that require nutritional coenzymes, most notably folate—a B vitamin that serves as a one-carbon carrier/donor, and also vitamins B12, B6, and B2 and methionine. Disruptions in one-carbon metabolism due to deficiency of the nutrients or genetic polymorphisms of the enzymes involved have been linked to cancer etiology through insufficient DNA synthesis/repair and aberrant gene expression.[2]

Epidemiologic evidence linking genetic susceptibility in one-carbon metabolism to cancer risk is most extensive for colorectum[7] and growing for cancers of breast,[8] stomach,[9] and other sites. Non-Hodgkin lymphoma (NHL), like other cancers, exhibits genetic instability[10] and aberrant DNA methylation patterns,[11] suggesting a key role of one-carbon metabolism in lymphoid tissues with high turnover rates and supporting its involvement in lymphomagenesis. In fact, genetic polymorphisms of some one-carbon metabolism enzymes have been associated with an altered risk of adult lymphomas[12-20] and leukemias[15,21-23]: lower risk was associated with *MTHFR* Ex5+79C>T (commonly known as 677C>T)[15,17,21] and Ex8-62A>C (1298A>C)[21] (or the combination[15,17,21]), *MTR* Ex26-20A>G (2756A>G),[14,16,20] *MTRR* Ex2-64A>G (66A>G),[15] *SHMT1* Ex12+138C>T (1420C>T),[13,22] and *TYMS* variants of variable number tandem repeats (VNTRs) Ex1+52-28base (3R versus 2R),[13,19,22] 494 6-bp deletion,[18] IVS6-68 C>T,[18] 1053C>T,[18] and their haplotypes.[18] Also, potential gene-gene interactions have been suggested.[13,15,20,22]

However, studies to date have considered only a few of these enzymes out of many involved in the metabolic pathway (Figure 1). Also, findings on these genetic variants have not been consistent in all studies: some studies showed conflicting findings for *MTHFR* Ex8-62A>C or the combination of its 2 single nucleotide polymorphisms (SNPs),[20] *MTR* Ex26-20A>G[12,17] and *TYMS* VNTR Ex1+52-28base,[20] and some showed null results for *CBS*,[16] *MTHFR*,[12,14-16,18,19] *MTR*,[15,18,22] and *SHMT1*.[18,19] This inconsistency in past studies may be in part due to the small size of most studies or to unavailability of dietary information to examine potential gene-nutrient interactions that have been reported for other cancers.[24-26]

In a US population–based case-control study, we previously reported that the highest quartiles of vitamin B6 and methionine intake were associated with about 50% lower risk of NHL overall and that folate intake was inversely associated with diffuse large

Figure 21 Introduction (Lim et al 2006)

6.3.3 Methods

Item 4: Case – Control. The study desing is early in the paper, in the end of the Introduction which is aceptable by Vandenbroucke et al., 2007.

B-cell lymphoma (DLBCL).[27] In this report, we investigated genetic susceptibility of selected one-carbon metabolism enzymes and their interaction with diet using a comprehensive assessment of the metabolic pathways (Figure 1).

Materials and methods

Study population

As described in detail previously,[28] newly-diagnosed and human immunodeficiency virus (HIV)–negative NHL cases were identified from 4 Surveillance, Epidemiology, and End Results (SEER) registries among women and men aged 20 to 74 years during the period of July 1998 through June 2000. On all NHL cases from local SEER registries, we obtained pathology and subtype information that was based on abstracted reports of the diagnosing pathologists. We then classified the histologically confirmed cases into subtypes of DLBCL, follicular lymphoma, and small lymphocytic lymphoma (SLL) according to the International Classification of Diseases-Oncology (ICD-O-2; codes for NHL: 9590-9595, 9670-9717).[29] Although pathology samples were not reviewed centrally, we consider pathological diagnosis of these main NHL subtypes to be reliable and comparable across SEER study centers.[30,31] Population controls who were aged 20 to 74 years, HIV negative, and with no history of NHL were identified among the study area residents via random-digit-dialing and Health Care Financing Administration (Medicare) files. The study was approved by the human subjects review boards at all participating institutions (NCI and SEER centers of Detroit, Iowa, Los Angeles, and Seattle), and we obtained written informed consent from all participants, in accordance with the Declaration of Helsinki.

We identified 2248 potentially eligible cases and interviewed 1321 cases (participation rate, 76%; response rate, 59%)[28]: we did not contact 520 cases (death, inability to locate, physician refusal, or relocation outside of the study area) and could not acquire participation of 407 cases. Of 2409 potentially eligible controls frequency matched to cases on sex, age, race, and SEER centers, 2046 were contacted, and 1057 were interviewed (participation rate, 52%; response rate, 44%). Among the interviewed participants, 1172 cases and 982 controls provided biologic samples for genotyping[32]: 773 cases and 668 controls provided blood samples, and 399 cases and 314 controls provided mouthwash buccal cell samples. Demographic characteristics (age, sex, education) were comparable among individuals who provided blood, buccal samples, or neither.[32] Among the 1141 cases and 949 controls who were genotyped successfully, 517 cases and 434 controls (about 50% of non–African American participants) were queried for diet/lifestyle history using a split-sample design described earlier,[27] of which 386 cases (75%) and 319 controls (74%) returned the questionnaire.

Genotyping

We considered enzymes and proteins involved in folate absorption and transport or intracellular one-carbon metabolism (Figure 1) and chose 14 genes that have been previously studied or are otherwise believed to play an important role in one-carbon metabolism.[3] In addition, we included 4 DNA repair genes (*MBD2, MGMT, MLH1,* and *MSH2)* that in part depend on one-carbon supplies, and therefore, may have a synergistic effect with nutritional or genetic factors of one-carbon metabolism on carcinogenesis. Twenty-nine SNPs and one insertion polymorphism in these genes were selected (Table 1), based on prior functional data from previous reports[18,22,34-37] or expected functional consequences in that the polymorphisms result in amino acid change or they are located within the 3′ untranslated region (UTR), which contains regulatory sequences and binding sites for other molecules that could alter the stability of the mRNA transcript of the gene.

Genotyping details are available elsewhere.[32] We extracted DNA using Puregene Autopure DNA extraction kits (Gentra Systems, Minneapolis, MN) from buffy coats and using phenol chloroform extraction methods for buccal cell samples.[38] All genotyping was conducted at the NCI Core Genotyping Facility (CGF, Advanced Technology Corporation, Gaithersburg, MD), using the Taqman platform (http://snp500cancer.nci.nih.gov; National Cancer Institute).[39] In order to conserve DNA, we genotyped the blood-based DNA samples first (~ 75%) and expanded to buccal cell samples if the blood-based DNA data yielded significant ($P < .05$) or suggestive (ie, borderline significance or significant linear trend) associations with NHL. As a result, 16 polymorphisms from 11 genes were not genotyped in buccal cell–based DNA due to null results in blood-based DNA (noted in Table 1). Genotype frequencies in general were similar for individuals who provided blood versus buccal cells and also similar by participation status.[40]

Genotyping was successful in 96% to 100% of DNA samples, similarly in blood- and buccal cell–based samples. Only 3 SNPs were not in Hardy-Weinberg equilibrium in white controls (*CBS* Ex9+33C>T, *MSH2* Ex6+23G>A, and *MTR* Ex26-20A>G; Table 1). To assure quality control, 40 replicate samples from 2 blood donors and duplicate samples from 100 study subjects were interspersed blindly among study samples for all genotyping, which yielded an agreement of 99% or more.[32]

Dietary assessment

Dietary intake was assessed using a modified version of the self-administered Block food frequency questionnaire.[27] Participants were queried on 107 food and beverage items for their "usual eating habits (as an

Figure 22 Material and Methods (Lim et al 2006)

Item 5: Population: women and men aged 20 to 74 years. Period: July 1998 through June 2000. On all NHL cases from local SEER registries classified the histologically confirmed cases into subtypes of DLBCL, follicular lymphoma, and small lymphocytic lymphoma (SLL) according to the International Classification of Diseases-Oncology. Population controls who were aged 20 to 74 years, HIV negative, and with no history of NHL. Location: NCI and SEER centers of Detroit, Iowa, Los Angeles, and Seattle

Item 6: There is not any information on the criteria and methods for selection of subsets of participants from a larger study. Population controls who were aged 20 to 74 years, HIV negative, and with no history of NHL were identified among the study area residents via random-digit-dialing and Health Care Financing Administration (Medicare) file. The cases were newly-diagnosed and human immunodeficiency virus (HIV)–negative NHL cases were identified from 4 Surveillance, Epidemiology, and End Results (SEER). The controls frequency matched to cases on sex, age and race but not study centers, to have the most parsimonious estimates. No information about number per case. We adjusted for the matching factors of age (continuous), sex, and race, but not study centers, to have the most parsimonious estimates.

Item 7a: They investigated genetic susceptibility of selected one-carbon metabolism enzymes and their interaction with diet using a comprehensive assessment of the metabolic pathways.

Item 7b: The genetic variant using the widely used nomenclature. All nutrients except alcohol were adjusted for total energy intake by the nutrient-density method. Regression models of folate, vitamin B6, and methionine were adjusted for each other for potential confounding. Additional adjustments for energy intake and other nutrients did not materially change the risk estimates.

Item 8a: There is information about genotyping is available by Wang et al., 2006 and dietary assessment.

Item 8b: Describe the laboratory method, include source and storage of DNA, genotyping method and platform. No any other information about call rates, error rates and state of laboratory.

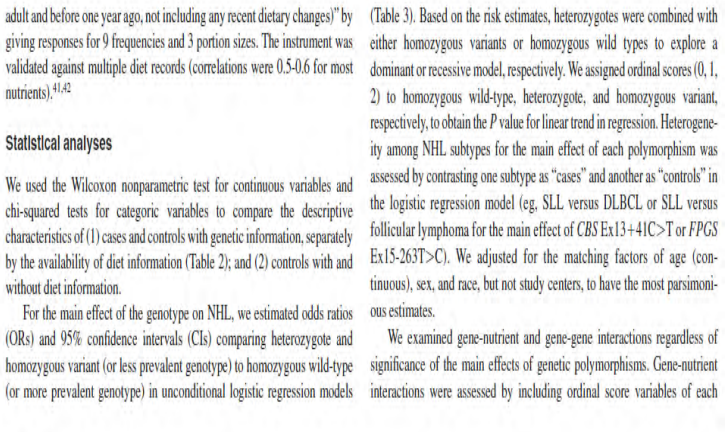
Item 9a: Our findings based on post diagnostic assessment of diet need to be replicated in prospective data with less influence of potential bias associated with recalled diet and selective participation of healthier controls. Although this is one of the largest polymorphism studies to date regarding one-carbon metabolism, it was still limited to examine gene-nutrient interactions.

Item 9b: No information.

Item 10: No information about study size.

Item 11: No information about treatments.

Item 12a: SAS/Genetics (version 9.1.3; SAS Institute, Cary, NC) to assess Hardy-Weinberg equilibrium and linkage disequilibrium among SNPs from the same gene.



adult and before one year ago, not including any recent dietary changes)" by giving responses for 9 frequencies and 3 portion sizes. The instrument was validated against multiple diet records (correlations were 0.5-0.6 for most nutrients).[41,42]

Statistical analyses

We used the Wilcoxon nonparametric test for continuous variables and chi-squared tests for categoric variables to compare the descriptive characteristics of (1) cases and controls with genetic information, separately by the availability of diet information (Table 2); and (2) controls with and without diet information.

For the main effect of the genotype on NHL, we estimated odds ratios (ORs) and 95% confidence intervals (CIs) comparing heterozygote and homozygous variant (or less prevalent genotype) to homozygous wild-type (or more prevalent genotype) in unconditional logistic regression models (Table 3). Based on the risk estimates, heterozygotes were combined with either homozygous variants or homozygous wild types to explore a dominant or recessive model, respectively. We assigned ordinal scores (0, 1, 2) to homozygous wild-type, heterozygote, and homozygous variant, respectively, to obtain the *P* value for linear trend in regression. Heterogeneity among NHL subtypes for the main effect of each polymorphism was assessed by contrasting one subtype as "cases" and another as "controls" in the logistic regression model (eg, SLL versus DLBCL or SLL versus follicular lymphoma for the main effect of *CBS* Ex13+41C>T or *FPGS* Ex15-263T>C). We adjusted for the matching factors of age (continuous), sex, and race, but not study centers, to have the most parsimonious estimates.

We examined gene-nutrient and gene-gene interactions regardless of significance of the main effects of genetic polymorphisms. Gene-nutrient interactions were assessed by including ordinal score variables of each

EFigure 23 Statistical analysis (Lim et al 2006)

Item 12b: Wilcoxon nonparametric test for continuous variables and chi-squared tests for categoric variables to compare the descriptive characteristics of cases and controls with genetic information, separately by the availability of diet information, controls with and without diet information. And comparing heterozygote and homozygous variant (or less prevalent genotype) to homozygous wild-type (or more prevalent genotype) in logistic regression models

Item 12 c: All participants who were genotyped and the subgroup who had both genotype and diet information.

Item 12d: Controls selected to be match the age and sex of the cases.

Item 12e: No information of any sensitivity analysis.

Item 12f and g: Hardy-Weinberg equilibrium and linkage disequilibrium among SNPs from the same gene. We explored haplotype analyses for genes with or more polymorphisms but did not detect any stronger associations, and therefore, present the findings from individual polymorphism analyses.

Item 12h: No information

Item 12i: To evaluate the probability of false-positive associations, we computed the false discovery rate (FDR), which controls the proportion of false positives out of all significant findings using the P values from the regression of score variables (ie, the trend test, also referred to as the additive model) using SAS software: we considered FDR less than 0.2 as noteworthy.

Item 12j: We also computed the false-positive report probabilities (FPRPs)45 using prior probabilities ranging from 0.1 to 0.001 based on gene selection criteria described in "Genotyping" and considered values below a criterion of 0.2 noteworthy as recommended in the initial description of the method.

6.3.4 Results

Item 13: Genotyping was successful in 96% to 100% of DNA samples, similarly in blood- and buccal cell–based samples. From 1321 and 1057, the 1141 cases and the 949 controls who were genotyped successfully. There is not any information about non participation at each stage and flow diagram.

Item 14a: Table

Item 14b: From 1321 and 1057, the 1141 cases and the 949 controls who were genotyped successfully.

Item 15: Table 1

Item 16a: However, we did not observe any independent interaction of folate when simultaneously adjusted for vitamin B6. We adjusted for the matching factors of age (continuous), sex, and race, but not study centers, to have the most parsimonious estimates. 95% confidence interval. All nutrients except alcohol were adjusted for total energy intake by the nutrient-density method. The main associations and gene-nutrient interactions were not confounded by other risk factors in combined and individual subtype analyses.

Item 16c: No information

Item 16d: Additional adjustments for energy intake and other nutrients did not materially change the risk estimate.(??? is thiw from a amultiple analysis)

Item 17a: All participants who were genotyped and the subgroup who had both genotype and diet information. The genotype-NHL association of polymorphisms that exhibited a significant main effect, linear trend, or interaction with a nutrient among people with both genotype and diet data. Main effects among all genotyped data (Table S2) showed similar association patterns and identified additional variants in FTHFD, MTHFS, and MTR for significant associations or trends with NHL. We examined interactions between all genetic variants and nutritional determinants,

specifically vitamin B6 and methionine that were previously found protective against NHL.27 We detected a number of significant interactions.

Item 17b: The results were summarized in a table.

Item 17c: No information.

6.2.5 Discussion

Item 18: The discussion summarized key results.

Item 19: The findings of gene-nutrient interactions have similar limitations that were discussed in our previous study of dietary associations. The population-based design of this case-control study might have reduced selection bias compared with hospital-based recruitment. The population-based design of this case-control study might have reduced selection bias compared with hospital-based recruitment. Our findings based on post diagnostic assessment of diet need to be replicated in prospective data with less influence of potential bias associated with recalled diet and selective participation of healthier controls.

Item 20: Our study is the first to show an interaction between a variant in *MTHFR* and one-carbon nutrients, specifically methionine, in relation to NHL. This finding emulates the previous epidemiologic reports of such interactions in colorectal cancer.

Item 21: Lastly, this line of investigation could also have implications for prognosis and survival of NHL. However, these results require replication in further large studies as well as pooled analyses.

Item 22: Conflict-of-interest disclosure: The authors declare no competing financial interests.

6.4 Title: Polymorphisms in xenobiotic-metabolizing genes and the risk of chronic lyphocytic leykemia and non Hodghin's lymphoma in adult Russian patients

Authors: Olga A. Gra, Andrey S. Glotov, Eugene A. Nikitin, Oleg S. Glotov, Viktoria E. Kuznetsova, Alexander V. Chudinov, Andrey B. Sudarikov, and Tatyana V. Nasedkina

Am J Hematol. 2008;83(4):279–287.

6.4.1 Title and Abstract

Item 1a: There is not the usual term in the title or the abstract

.

Item 1b: There is a balanced summary because is not separate the background, design, patients, measurements, results, limitations and conclusions.

Polymorphisms in xenobiotic-metabolizing genes and the risk of chronic lymphocytic leukemia and non-Hodgkin's lymphoma in adult Russian patients

Olga A. Gra,[1] Andrey S. Glotov,[1,2] Eugene A. Nikitin,[3] Oleg S. Glotov,[2] Viktoria E. Kuznetsova,[1] Alexander V. Chudinov,[1] Andrey B. Sudarikov,[3] and Tatyana V. Nasedkina[1*]

Polymorphisms in genes coding xenobiotic-metabolizing enzymes are considered as risk factors modifying susceptibility to cancer. We developed a biochip for the analysis of 18 mutations in 10 genes of metabolizing system: *CYP1A1*, *CYP2D6*, *GSTT1*, *GSTM1*, *MTHFR*, *MTRR*, *NQO1*, *CYP2C9*, *CYP2C19*, and *NAT2*. Using allele-specific hybridization on the biochip 76 T-cell non-Hodgkin's lymphoma (NHL) patients, 83 B-cell chronic lymphocytic leukemia (B-CLL) patients, and 177 healthy donors were tested. Polymorphic *CYP1A1* alleles were more frequent in B-CLL patients relative to normal controls, for example, a combination of polymorphic variants 4887C > A, 4889A > G, and 6235T > C (OR = 1.76, 95% CI = 1.0–3.1). The *GSTM1* null genotype was more frequent in NHL patients relative to controls (OR = 1.82, 95% CI = 1.1–3.1). The combination of unfavorable polymorphic *CYP1A1* variants and *GSTM1* null genotype was found more frequently in B-CLL patients relative to controls (OR = 2.52, 95% CI = 1.3–4.9). In addition, male B-CLL patients demonstrated a significantly increased occurrence of heterozygous and homozygous allele **2* of *CYP2C9* gene (OR = 2.38, 95% CI = 1.1–5.2) as well as a combination of alleles **2* and **3* of the gene (OR = 2.09, 95% CI = 1.1–3.9). Thus, our findings show the association between polymorphic alleles of *CYP1A1*, *GSTM1*, and *CYP2C9* genes and the risk to develop NHL or B-CLL. The developed biochip can be considered as a convenient analytical tool for research studies and predictive analysis in oncohematology. Am. J. Hematol. 83:279–287, 2008.

Figure 24 Abstract (Gra et al.2007)

6.4.2 Introduction

Item 2: There is scientific background of the study that provides important context for readers. (Vandenbroucke et al., 2007)

Item 3: There is not any information if this is the first report of a genetic association in CLL.

Introduction

Genetic susceptibility studies of lymphoproliferative disorders may serve to identify at risk populations and to clarify important disease mechanisms. B-cell chronic lymphocytic leukemia (B-CLL) is the most common leukemia amounting to about 30% of cases in adults [1,2]. Non-Hodgkin lymphoma (NHL) is a group of histologically and biologically heterogeneous malignant lymphoid neoplasms of unknown etiology [3]. T-cell lymphomas account for 15–20% of NHL in Western countries, as well as in Russia. Over the last several decades the significant growth of incidence for B-CLL and NHL, has been observed, especially, in developed countries. This fact greatly stimulates searching for hereditary and environmental risk factors which may impact to the development of these malignancies. Familial aggregation has been well documented for both B-CLL and lymphomas, indicating the substantial contribution of hereditary factors to the development of disease. The risk to develop B-CLL was 3–7 folds higher among close relatives of CLL patients [4,5]. In the case of lymphomas, a family history of any hematopoietic malignancy was found to be associated with 2–3 fold increased risk of NHL [6,7]. Genome-wide linkage scans of CLL families revealed a number of chromosome bands with potential candidate genes, for example, 11p11 [8] and 13q22.1 [9]. The role and function of these candidate regions are under investigation now. But, it is very likely also, that common genetic variants, each with apparently minor effects on tumor phenotype, may influence disease susceptibility in hematological malignancies. It is especially true for lymphomas, where known risk factors like viral infections and dysfunctions of immune system account for only a small proportion of the total NHL cases [3,10].

To date, a number of case-control association studies have examined the role of genetic polymorphisms in the risk identified as potential susceptibility loci [11]. Positive associations have been found between polymorphic alleles of genes *ATM*, *BRCA2*, and *CHEK2* involved in the DNA damage-response and cell-cycle pathways and risk of B-CLL [12]. Among potential biomarkers increasing NHL risk two susceptibility alleles in TNF and IL10 have been identified [13]. Several B-CLL and NHL studies have examined polymorphisms involved in folate metabolism; although, results have been inconsistent [14–17]. Among low-penetrance candidate genes, those involved in xenobiotic metabolism draw a special attention, because they may provide clues to identify potential lymphomagens and to estimate carcinogen effects of environmental pollution. Published data indicate that xenobiotic-metabolizing polymorphic alleles in some populations correlate with increased risk of different lymphoproliferative diseases [18–22]. The majority of articles were devoted to the analysis of acute leukemia and, particularly, childhood leukemia [21,23–25]. Thus, it would be interesting to investigate associations between polymorphous variants

[1]Engelhardt Institute of Molecular Biology, Russian Academy of Sciences, Moscow, Russia; [2]Ott Research Institute of Obstetrics and Gynecology, Russian Academy of Medical Sciences, St. Petersburg, Russia; [3]National Hematology Research Center, Russian Academy of Medical Sciences, Moscow, Russia

Contract grant sponsor: Russian Foundation; Contract grant number: 06-04-49771.

*Correspondence to: Tatyana V. Nasedkina, EIMB, 32 Vavilov St., Moscow 119991, Russia. E-mail: nased@eimb.ru

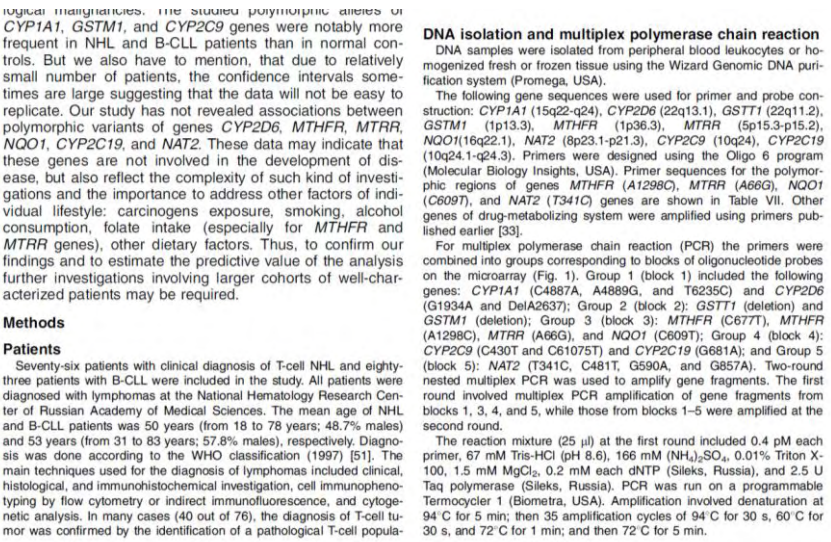
Received for publication 5 May 2006; Revised 10 October 2007; Accepted 15 October 2007

Am. J. Hematol. 83:279–287, 2008.

Published online 5 December 2007 in Wiley InterScience (www.interscience.

6.4.3 Methods

Figure 25 Introduction (Gra et al. 2007)



logical malignancies. The studied polymorphic alleles of *CYP1A1*, *GSTM1*, and *CYP2C9* genes were notably more frequent in NHL and B-CLL patients than in normal controls. But we also have to mention, that due to relatively small number of patients, the confidence intervals sometimes are large suggesting that the data will not be easy to replicate. Our study has not revealed associations between polymorphic variants of genes *CYP2D6*, *MTHFR*, *MTRR*, *NQO1*, *CYP2C19*, and *NAT2*. These data may indicate that these genes are not involved in the development of disease, but also reflect the complexity of such kind of investigations and the importance to address other factors of individual lifestyle: carcinogens exposure, smoking, alcohol consumption, folate intake (especially for *MTHFR* and *MTRR* genes), other dietary factors. Thus, to confirm our findings and to estimate the predictive value of the analysis further investigations involving larger cohorts of well-characterized patients may be required.

Methods

Patients

Seventy-six patients with clinical diagnosis of T-cell NHL and eighty-three patients with B-CLL were included in the study. All patients were diagnosed with lymphomas at the National Hematology Research Center of Russian Academy of Medical Sciences. The mean age of NHL and B-CLL patients was 50 years (from 18 to 78 years; 48.7% males) and 53 years (from 31 to 83 years; 57.8% males), respectively. Diagnosis was done according to the WHO classification (1997) [51]. The main techniques used for the diagnosis of lymphomas included clinical, histological, and immunohistochemical investigation, cell immunophenotyping by flow cytometry or indirect immunofluorescence, and cytogenetic analysis. In many cases (40 out of 76), the diagnosis of T-cell tumor was confirmed by the identification of a pathological T-cell popula-

DNA isolation and multiplex polymerase chain reaction

DNA samples were isolated from peripheral blood leukocytes or homogenized fresh or frozen tissue using the Wizard Genomic DNA purification system (Promega, USA).

The following gene sequences were used for primer and probe construction: *CYP1A1* (15q22-q24), *CYP2D6* (22q13.1), *GSTT1* (22q11.2), *GSTM1* (1p13.3), *MTHFR* (1p36.3), *MTRR* (5p15.3-p15.2), *NQO1*(16q22.1), *NAT2* (8p23.1-p21.3), *CYP2C9* (10q24), *CYP2C19* (10q24.1-q24.3). Primers were designed using the Oligo 6 program (Molecular Biology Insights, USA). Primer sequences for the polymorphic regions of genes *MTHFR* (*A1298C*), *MTRR* (*A66G*), *NQO1* (*C609T*), and *NAT2* (*T341C*) genes are shown in Table VII. Other genes of drug-metabolizing system were amplified using primers published earlier [33].

For multiplex polymerase chain reaction (PCR) the primers were combined into groups corresponding to blocks of oligonucleotide probes on the microarray (Fig. 1). Group 1 (block 1) included the following genes: *CYP1A1* (C4887A, A4889G, and T6235C) and *CYP2D6* (G1934A and DelA2637); Group 2 (block 2): *GSTT1* (deletion) and *GSTM1* (deletion); Group 3 (block 3): *MTHFR* (C677T), *MTHFR* (A1298C), *MTRR* (A66G), and *NQO1* (C609T); Group 4 (block 4): *CYP2C9* (C430T and C61075T) and *CYP2C19* (G681A); and Group 5 (block 5): *NAT2* (T341C, C481T, G590A, and G857A). Two-round nested multiplex PCR was used to amplify gene fragments. The first round involved multiplex PCR amplification of gene fragments from blocks 1, 3, 4, and 5, while those from blocks 1–5 were amplified at the second round.

The reaction mixture (25 μl) at the first round included 0.4 pM each primer, 67 mM Tris-HCl (pH 8.6), 166 mM $(NH_4)_2SO_4$, 0.01% Triton X-100, 1.5 mM $MgCl_2$, 0.2 mM each dNTP (Sileks, Russia), and 2.5 U Taq polymerase (Sileks, Russia). PCR was run on a programmable Termocycler 1 (Biometra, USA). Amplification involved denaturation at 94°C for 5 min; then 35 amplification cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 1 min; and then 72°C for 5 min.

Figure 26 Methods (Gra et al.2007)

Item 4: No information about study design early in the paper.

Item 5: Seventy-six patients with clinical diagnosis of T-cell NHL and eighty three patients with B-CLL were included in the study. All patients were diagnosed with lymphomas at the National Hematology Research Center of Russian Academy of Medical Sciences. Diagnosis was done according to the WHO classification (1997). The control group included 177 healthy donors. Blood was taken at the blood transfusion center of the National Hematology Research Center. All patients and healthy donors were residents of European part of Russia.

Item 6: There is not any information on the criteria and methods for selection of subsets of participants from a larger study.

Item 7a: Association between main polymorphic genetic variants of metabolizing enzymes, which process environmental carcinogens or play key role in intracellular methylation, and individual susceptibility to such malignant diseases as T-cell NHL and B-CLL in a Russian population.

Item 7b: All patients and healthy donors were residents of European part of Russia.

Item 8a: The details of methods of assessment.

Item 8b: Describe the laboratory method, include source and storage of DNA, genotyping method and platform. There is not information about error rates, call rates, state of laboratory.

Item 9a: No information.

Item 9b: Not information about a potential bias.

Item 10: Not information about study size.

Item 11: No information about treatment.

Item 12a: GraphPad InStat software, USA,

Item 12b: Hardy-Weinberg equilibrium was tested using the standard X^2 test, Fisher test

Item 12 c: Not information about missing data

Statistical analysis

The fit of the genotype frequencies to the expected distribution in Hardy-Weinberg equilibrium was tested using the standard χ^2 test (GraphPad InStat software, USA). The genotype frequencies for all studied biotransformation system genes corresponded to the Hardy-Weinberg distribution (the highest χ^2 was 2.1305; $P > 0.05$). Pairwise comparison of allelic and genotypic frequencies and evaluation of the relationship between the alleles and risk of NHL and B-CLL relied on two-sided exact Fisher test. The odds ratio (OR) of the disease was calculated for particular genotypes using the following equation:

$$\mathrm{OR} = \frac{a/b}{c/d},$$

where $a = n_1$, $b = N_1 - n_1$, $c = n_2$, $d = N_2 - n_2$, N_1 and N_2 are sample size, n_1 and n_2 are numbers of individuals with the studied character in these two samples. OR values were computed in this work using a 95% confidence interval (95% CI). The threshold for rejecting the null hypothesis was set to 5%.

Figure 27 Statistical analysis (Gra et al.2007)

Item 12d: No information.

Item 12e: No information of any sensitivity analysis.

Item 12f: The controls were tested for HWE.

Item 12g: No information.

Item 12h: No information.

Item 12i: No information.

Item 12j: No information.

6.4.4 Results

Item 13: 83 B-CLL and 76 cases T-CLL and 177 controls. No information about missing data. No information about no participation at each stage and flow diagram.

Results

The genotypes were assigned according to fluorescent signal patterns obtained in hybridization experiments using biochip. The scheme of biochip is shown in Figure 1. Figure 2 exemplifies a biochip hybridization pattern for a sample with the following genotype: *CYP1A1* (**4/*1*), *CYP2D6* (**4/*1*), *GSTT1* (+/+), *GSTM1* (+/+), *MTHFR* (*C/T; A/C*), *MTRR* (*A/G*), *NQO1* (**1/*1*), *CYP2C9* (**2/*1*), *CYP2C19* (**1/*1*), and *NAT2* (*S2/S2*). The correctness of biochip analysis was confirmed in experiments with 35 control samples of known genotype.

Distribution of metabolizing genes genotypes

The distribution of *CYP1A1*, *CYP2D6*, *GSTT1*, *GSTM1*, *MTHFR*, *MTRR*, *NQO1*, *CYP2C9*, *CYP2C19*, and *NAT2* genotypes in NHL (n = 76), B-CLL (n = 83) patients and healthy controls (n = 177) is given in Table I. No statistically significant difference in polymorphic genotype frequencies of *CYP2D6*, *MTHFR*, *MTRR*, *NQO1*, *GSTT1*, *CYP2C19*, and *NAT2* genes have been revealed between T-cell NHL or B-CLL patients and normal controls. Both NHL and B-CLL patients demonstrated higher incidence of the *GSTM1* null genotype relative to normal controls (Table I). The difference was significant for NHL patients (OR = 1.82, 95% CI = 1.05–3.14, P = 0.0395). For *CYP2C9* gene the higher incidence of **2/*3* genotype in B-CLL patients relative to normal controls have been found and the difference is statistically significant (OR = 9.48, 95% CI = 1.03–86.85, P = 0.0329).

Figure 28 Results (Gra et al. 2007)

Item 14: Table. No information about missing data.

Item 15: Table

Item 16a: 95% confidence interval. No information about confounders or adjustment.

Item 16b: No information

Item 16c: No information

Item 16d: No information

Item 17a: Distribution of metabolizing genes genotypes. Combined analysis for CYP1A1 and GSTM1 loci. Sex differences in the occurrence of polymorphic variants of the metabolizing system genes.

Item 17b: The results were summarized in a table.

Item 17c: No information

TABLE I. Distribution of Genotypes of Xenobiotic-Metabolizing Genes

	NHL (n = 76)			B-CLL (n = 83)			
Allele/genotype	n (%)	OR (95% CI)	P	n (%)	OR (95% CI)	P	Controls (n = 177), n (%)
CYP1A1							
**1/*1*	53 (69.7)	1		53 (63.9)	1		134 (75.7)
**1/*2A*	10 (13.2)	1.4 (0.6–3.2)	0.51	11 (13.3)	1.5 (0.7–3.5)	0.28	18 (10.2)
**1/*2B*	9 (11.8)	1.6 (0.7–4.0)	0.33	12 (14.4)	2.2 (0.9–5.0)	0.07	14 (7.9)
**1/*4*	3 (4.0)	0.8 (0.2–3.2)	1.00	7 (8.4)	2.0 (0.7–5.6)	0.25	9 (5.1)
**2A/*2A*	0 (0.0)	–	–	0 (0.0)	–	–	1 (0.55)
**2B/*4*	1 (1.3)	–	–	0 (0.0)	–	–	0 (0.0)
**4/*4*	0 (0.0)	–	–	0 (0.0)	–	–	1 (0.55)
CYP2D6							
**1/*1*	52 (68.4)	1		55 (66.3)	1		116 (65.6)
**1/*3*	1 (1.3)	0.7 (0.1–7.3)	1.00	1 (1.2)	0.7 (0.1–6.9)	1.00	3 (1.7)
**1/*4*	20 (26.3)	0.9 (0.5–1.7)	0.88	24 (28.9)	1.1 (0.6–1.9)	0.88	48 (27.1)
**3/*3*	0 (0.0)	–	–	0 (0.0)	–	–	1 (0.55)
**3/*4*	0 (0.0)	–	–	0 (0.0)	–	–	1 (0.55)
**4/*4*	3 (4.0)	0.8 (0.2–3.3)	1.00	3 (3.6)	0.8 (0.2–3.1)	0.79	8 (4.5)
GSTT1							
Present	56 (73.5)	1		58 (70.0)	1		126 (71.2)
Null	20 (26.5)	0.9 (0.5–1.6)	0.76	25 (30.0)	1.1 (0.6–1.9)	0.88	51 (28.8)
GSTM1							
Present	30 (39.5)	1		38 (46.0)	1		96 (54.2)
Null	46 (60.5)	1.8 (1.1–3.1)	0.04	45 (54.0)	1.4 (0.8–2.4)	0.23	81 (45.8)
MTHFR C677T							
CC	39 (51.3)	1		44 (53.0)	1		85 (48.0)
CT	28 (36.9)	0.8 (0.4–1.4)	0.39	35 (42.2)	0.9 (0.5–1.5)	0.59	79 (44.6)
TT	9 (11.8)	1.5 (0.6–3.8)	0.46	4 (4.8)	0.6 (0.2–1.9)	0.58	13 (7.4)
MTHFR 1298C							
AA	36 (47.4)	1		39 (47.0)	1		81 (45.8)
AC	30 (39.5)	0.8 (0.5–1.5)	0.56	38 (45.8)	1.0 (0.6–1.7)	1.00	82 (46.3)
CC	10 (13.1)	1.6 (0.7–4.0)	0.34	6 (7.2)	0.9 (0.3–2.5)	1.00	14 (7.9)
MTRR A66G							

Figure 29 Table (Gra et al. 2007)

6.4.5 Discussion

Item 18: The article summarize key results by gene.

Item 19: No information

Item 20: Provide no statistically significant evidence that MTHFR plays a role in development of CLL, we cannot entirely exclude a small effect in risk of CLL associated with MTHFR variants. Moreover, as there is evidence of a gene-environment effect with respect to folate metabolism and cancer risk, it is possible that polymorphisms of MTHFR may mediate CLL risk in the context of a low-folate diet.

Item 21: No information

Discussion

It is generally accepted that exogenous carcinogens are metabolized in human organism with the formation of active metabolites, binding and damaging nuclear and mitochondrial DNA [18–20], so the exposure to cytotoxic chemicals capable to cause oxidative stress may contribute to the development of many diseases including cancer. Oxidative metabolism of xenobiotics is mediated by phase I enzymes, members of the cytochrome P450 superfamily (CYP1A1, CYP2D6, CYP2C9, and CYP2C19). Cytochromes P450, mainly localized in the liver, use singlet oxygen to activate xenobiotics, thus transforming them into highly active intermediate metabolites [19]. Intermediate genotoxic metabolites are substrates for subsequent detoxification mediated by phase II enzymes such as glutathione *S*-transferases

Figure 29 Discussion (Gra et al. 2007)

Item 22: No information about conflicts of interest.