Research Article – AUTHOR COPY – Article published in Dermatology (2022): https://doi.org/10.1159/000520941

Dark-Green Leafy Vegetable Intake, MTHFR Genotype, And Risk Of Cutaneous Squamous Cell Carcinoma

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Short title: Green leafy vegetable intake, MTHFR and SCC

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Key message: The protective association between green-leafy vegetable consumption and SCC depends on folate metabolism genes

Number of tables: 1

Number of figures: 0

Word count: 1,504

Keywords: Skin cancer, squamous cell carcinoma, folate, diet, methylenetetrahydrofolate reductase (MTHFR)

Abstract

Background: Evidence suggests that consumption of dark green leafy vegetables may influence decrease the risk of cutaneous squamous cell carcinoma (SCC). Dark-green leafy vegetables contain folate as a main component among other nutrients, thus we hypothesised that their possible observed protective effect on SCC observed in previous studies, would be more evident in persons with specific genotypes related to folate metabolism. ***Methods***: Genotyping of methylenetetrahydrofolate reductase (MTHFR) gene variants rs18011331 (C677T) and rs1801131 (A1298C) was carried out for 1,128 participants in an Australian community-based longitudinal study of skin cancer. Dietary intakes were assessed through repeated Food Frequency Questionnaires (1992-1996) and all incident skin cancers were recorded 1992-2007 and histologically confirmed. We assessed associations between intake of dark-green leafy vegetables and SCC development in strata defined by genotype, by calculating relative risks (RRs) with 95% confidence intervals (CIs) using generalized linear models with negative binomial distribution and person-years of follow-up as offset. ***Results***: High vs. low intake of dark-green leafy vegetables was associated with lower risk of SCC tumours in carriers of the C677T variant allele (RR=0.42, 95% CI= 0.23-0.75), and within wild-type A1298C homozygotes (RR=0.43, 95% CI= 0.22-0.85). ***Conclusion***: The protective effect of dark-green leafy vegetables on cutaneous SCC may be genotype-dependent. Folate metabolism-related gene polymorphisms should be considered when assessing the relation of green leafy vegetables to cancer risk.

Introduction

Cutaneous squamous cell carcinoma (SCC) is the second most common type of skin cancer in fair-skinned populations worldwide. While exposure to ultraviolet (UV) radiation is the most important risk factor [1], dietary factors also influence their development [2].

From our longitudinal study of skin cancer in Australian adults, we previously showed that a dietary pattern characterised by vegetable and fruit intake protects against SCC [3], which was particularly explained by consumption of green-leafy vegetables [4, 3]. Green leafy vegetables are abundant dietary sources of several nutrients, including calcium, magnesium, iron, provitamin A carotenoids (β-carotene, α-carotene, γ-carotene, β-cryptoxanthin) and other carotenoids, vitamins C, E, and K, and B-vitamins [5]. While a number of these nutrients have been inversely associated with SCC development [6, 7], we previously proposed that the protection afforded by green leafy vegetables (including spinach and silver beet) that we observed, may be due particularly to their high folate content. We also proposed that polymorphisms in genes that express folate-metabolising enzymes may modify this association [4] (Figure 1).

In particular polymorphisms in the gene that codes for the folate-metabolising enzyme, methylenetetrahydrofolate reductase (MTHFR), are associated with development of SCC [8, 9] and of other cancer types [10]. Homozygotes for the C677T variant (TT) only have 30% of MTHFR enzyme activity, and heterozygotes (CT) 65%, compared to (CC) homozygotes [10]. In a large US population-based study, homozygote variant carriers had an increased risk of SCC when their folate intake was low [8]. In a study of renal transplant patients, who are at high risk of developing cutaneous SCC, carriers of the MTHFR 677T allele had a substantially increased SCC risk compared to non-carriers [9].

Here we have tested the hypothesis that the observed protective effect of dark-green leafy vegetable consumption is strongest in carriers of the MTHFR 677T allele, that is, in persons with reduced MTHFR enzyme activity, compared to wild type. We also assessed the reported association between the A1298C MTHFR variant and skin cancer [11].

Materials and Methods

Participants were drawn from people enrolled in the Nambour Skin Cancer Prevention Trial (1992-1996) and subsequently followed-up for 11 years (1996-2007). In the Trial, 1,621 randomly-selected residents of the township of Nambour, Queensland, were randomised to daily vs discretionary sunscreen application and to beta-carotene supplements vs placebo[12] . Participants were fully characterised for skin phenotype, ongoing sun exposure and protection behaviours and other factors relevant to skin cancer, through regular questionnaires. Dietary intake data were collected in 1992, 1994, and 1996, using a 129-item validated Food Frequency Questionnaire (FFQ) as previously described [4]. In 1992, 1994, 1996, and 2007, dermatologists carried out full-body skin examinations of participants and clinically diagnosed skin cancers were histologically confirmed. Skin cancers histologically diagnosed in participants between skin examinations were captured by linkage with pathology laboratories. Blood samples used for genotyping were collected in 1996. This study was approved by the Human Research Ethics Committee of the QIMR Berghofer Medical Research Institute and all participants gave written informed consent.

Genotyping was carried out using Illumina Global Screening Array (GSA) chips (model GSAMD-24v1-0-20011747; manifest revision A4), in two batches under contract by the Human Genomics Facility (HuGeF) at Erasmus Medical Centre, Rotterdam. After quality control, there

were 1,127 participants with dietary intake and SNP data available, including 181 who developed SCC during follow-up. Genotypes for rs18011331 (C677T) and rs1801131 (A1298C) were analysed because these are the two most studied genotypes with relevance to folate metabolism.

The outcome was incidence of SCC tumours diagnosed after the baseline 1992 skin examination to 31 December 2007. SCC tumours and person-years of follow-up were counted until date of withdrawal from the study, date of death, or 31 December 2007, whichever came first.

We assessed whether intake of dark-green leafy vegetables (spinach and silverbeet) was associated with incident SCC within two strata defined by variant allele carrying status, namely wild type homozygotes, and variant allele homozygotes plus heterozygotes combined. Participants were ranked according to their estimated total daily intake of and divided into thirds: low (median 0 grams/day), medium (median 1.9 grams/day), or high (median 13.5 grams/day) intake. Relative risks (RR) with 95% confidence intervals (CIs) for groups with increasing dark-green leafy vegetable intakes compared with the lowest intake group were derived within genotype strata using generalized linear models with negative binomial distribution and person-years of follow-up as offset. Sensitivity analyses were performed by excluding first and second-degree relatives from the analyses.

Results

High intake of dark-green leafy vegetables was associated with a lower risk of SCC tumours in carriers of the C677T variant allele (RR=0.42, 95% CI= 0.23-0.75) but not in (CC) homozygotes (Table 1). High intake of dark-green leafy vegetables was also protective for SCC with wild-type (AA) carriers of the A1298C genotype (RR=0.43, 95% CI= 0.22-0.85; Table 1). Excluding first (n=106) and second-degree (n=22) relatives did not change these results.

Discussion/Conclusion

Our results show that relatively high intake of dark-green leafy vegetables may be especially protective of SCC tumours in persons who carry the variant C677T allele, but not in those with wild type for this SNP, thus confirming our prior hypothesis. However, the results also indicate a protective association of high dark-green leafy vegetable intake within the A1298C wild-type genotype group, not in carriers of the variant allele for that SNP. These study results combined with our previous findings [4] indicate that intake of dark-green leafy vegetables may afford protection not only in those at highest risk of cutaneous SCC, indicated by prior skin cancer history, but also according to MTHFR genotype. Although consumption of these vegetables may be correlated with intake of other vegetables, fruit, and with generally healthy lifestyles, we have previously shown that a protective effect on SCC of a dietary pattern characterised by high intakes of a variety of different fruits and vegetables, is particularly explained by intake of green leafy vegetables [3].

The mechanisms underlying a possible interaction between MTHFR C677T variants and intake of folate or folate-rich foods remain to be confirmed. On one hand, reduced activity of the MTHFR enzyme may cause aberrant DNA methylation, such as promotor hypermethylation of CDKN2A [9] or global DNA hypomethylation [13]. On the other hand, the C677T (TT) variant may prevent depletion of 5,10-methylenetetrahydrofolate, which the MTHFR enzyme converts to 5-methylene and similar tetrahydrofolates—with 5,10-methylenetetrahydrofolate being a cofactor for nucleotide synthesis and thus supporting DNA synthesis and repair, this mechanism could possibly explain the potential benefit of high folate intake in C677T (TT) variant carriers. There is some evidence in regard to SCC of the oesophagus that moderately high folate intake or

status may offset tumour-promoting effects of aberrant DNA methylation in p16 and p53 genes [14]. A recent National Institutes of Health expert panel recommended further research of the possible mediating effect that MTHFR genotypes may have in the mechanisms through which folate intake influences health outcomes [15].

Major strengths of this study include the excellent phenotypic characterisation of the study cohort over time and the assessment of dietary intake using a validated instrument. Limitations include the small sample size that prevented analyses of three genotype groups, and lack of data on intake of folic acid from supplements, or folate status. We have adjusted for sunscreen allocation during the Nambour skin cancer prevention trial, which tracked well with post-trial longer-term sunscreen use[16], but a possibility of residual confounding by sun protective behaviour remains.

In conclusion, our findings indicate that relatively high intake of dark-green leafy vegetables may afford protection against cutaneous SCC in carriers of the variant C677T allele, and in A1298C wild type carriers. Furthermore, they suggest that when assessing associations between consumption of dark-green leafy vegetables or folate intake and cancer, gene polymorphisms involved in folate metabolism should also be considered.

Acknowledgement

We thank Prof Lyn Griffiths, Ms Rebecca Grealy and Griffith University for their invaluable contributions to the Nambour Skin Cancer Study DNA resource, including sharing sample processing and storage. We are also grateful to Profs Nick Martin and David Whiteman for their support.

Statement of Ethics

This study was approved by the ethics committee of the QIMR Berghofer Medical Research Institute and all participants provided written informed consent.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

Funding Sources

This work was supported by a Program Grant (1073898) from the National Health and Medical Research Council of Australia (NHMRC).

Author Contributions

M.C.B.H., A.C.G, and J.v.d.P.: study design. M.C.B.H., A.A., A.J.R.-A., and U.E.L.: data analysis. M.C.B.H., A.A., A.J.R.-A., U.E.L., A.C.G, and J.v.d.P.: writing of the paper.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Table 1. Association between tertiles of dark-green leafy vegetable intake and tumour-based SCC incidence by MTHFR genotype

SNP	Genotype: Dark-green leafy vegetable intake	*N*	SCC tumors (*n*)	SCC persons (*n*)	Person-years	SCC incidence, Tumors per 100,000 PY	SCC incidence, Persons per 100,000 PY	Relative Risk[1] (95% CI)
rs1801133[2,3]	CC: LOW	166	51	29	2520.7	2023.22	1150.46	1.00
(C677T)	CC: MED	166	49	27	2544.1	1925.99	1061.26	1.08 (0.56-2.09)
	CC: HIGH	161	65	27	2471.9	2629.58	1092.29	0.72 (0.37-1.39)
	TT, TC: LOW	221	103	34	3447.6	2987.55	986.18	1.00
	TT, TC: MED	213	75	41	3266.7	2295.93	1255.11	0.94 (0.56-1.58)
	TT, TC: HIGH	200	37	23	3025.6	1222.91	760.19	0.42 (0.23-0.75)
rs1801131[3]	AA: LOW	192	87	28	3000.8	2899.25	933.09	1.00
(A1298C)	AA: MED	180	63	33	2757.7	2284.49	1196.64	1.14 (0.61-2.11)
	AA: HIGH	172	44	20	2616.1	1681.90	764.50	0.43 (0.22-0.85)
	CC, CA: LOW	195	67	35	2967.6	2257.72	1179.41	1.00
	CC, CA: MED	199	61	35	3053.1	1997.98	1146.38	0.89 (0.52-1.53)
	CC, CA: HIGH	190	58	30	2897.2	2001.96	1035.50	0.72 (0.42-1.24)

[1] Results from models of tertiles of dark-green leafy intake and SCC risk stratified by genotype and adjusted for age, sex, trial treatment allocation and GWAS batch, energy intake, smoking, clinical neck elastosis, use of any dietary supplement, skin colour, port and sherry intake [17]. [2] no data for n=1 participant. [3] P-value for interaction between genotype and dark-green leafy vegetable intake was 0.34 for rs1801133 and 0.65 for rs1801131 in the fully-adjusted model.

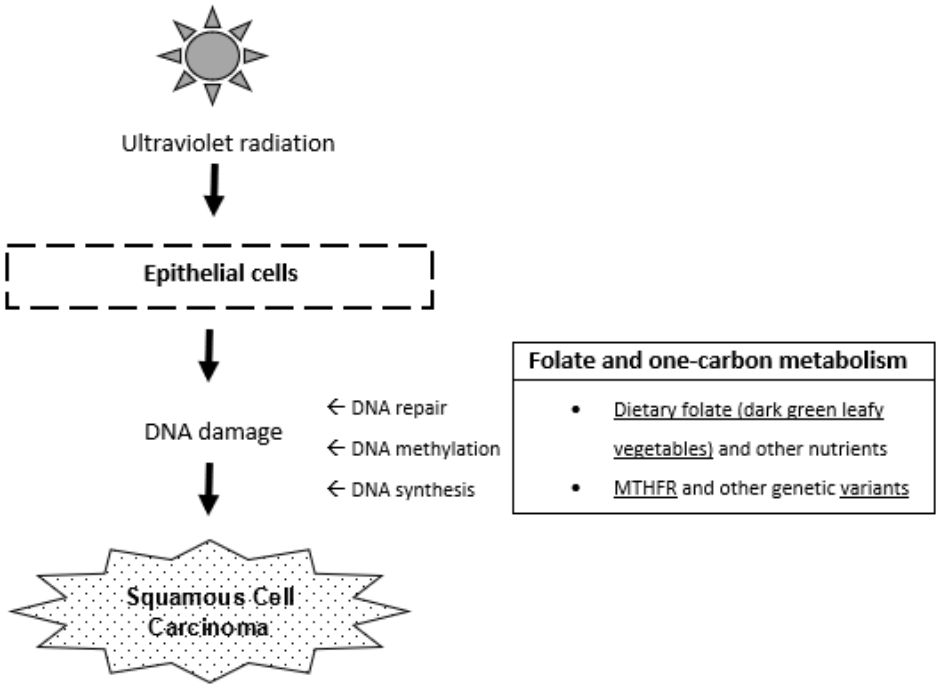

Figure 1. Suggested relationship between dark green leafy vegetable intake, MTHFR polymorphisms, and squamous cell carcinoma development.