Head and neck carcinogenesis: impact of MTHFD1 G1958A polymorphism

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Summary

Objective: To investigate the MTHFD1 G1958A polymorphism involved in the folate metabolism as a risk for head and neck cancer, and to find the association of the polymorphism with the risk factors and clinical and histopathological characteristics. **Methods:** Retrospective study investigating MTHFD1 G1958A polymorphism in 694 subjects (240 patients in the Case Group and 454 in the Control Group) by Restriction Fragment Length Polymorphism (RFLP) Analysis. Multiple logistic regression and chi-square tests were used in the statistical analysis. **Results:** Multivariable analysis showed that smoking and age over 42 years were disease predictors ($p < 0.05$). MTHFD1 1958GA or AA genotypes were associated with smoking ($p = 0.04$) and alcoholism ($p = 0.03$) and were more often found in more advanced stage tumors ($p = 0.04$) and in patients with a shorter survival ($p = 0.03$). **Conclusion:** The presence of MTHFD1 G1948A polymorphism associated with smoking and alcoholism raises the head and neck cancer risk.

Keywords: Polymorphism, genetic; head and neck neoplasms; alcoholism; smoking; methylenetetrahydrofolate dehydrogenase (NADP).

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Introduction

Head and neck neoplasms account for high incidence of deaths worldwide, being considered the sixth most common type[1]. The anatomical areas affected by these tumors include the oral cavity (40%), the pharynx (15%) and the larynx (25%)[2]. Data from the National Cancer Institute[2] showed there is a 3:1 male-female ratio and a higher incidence of oral cavity-located cases in the Brazilian population.

Head and neck cancer has smoking and alcoholism as its main risk factors[1]. Viral infections, especially with Epstein-Barr virus and human papillomavirus (HPV) subtypes 16 and 18, in addition to deficiencies or imbalances of vitamins and micronutrients, such as folic acid, vitamins A, C and E, zinc and selenium, were also associated with head and neck neoplasms occurrence[3-5].

Folate has a key role in oncology, mainly from its action on DNA methylation and purine and pyrimidine synthesis[6]. Genetic changes and deficiency of this vitamin show a relationship with cancer in several studies, including head and neck cancer[6-15].

The *Methylenetetrahydrofolate dehydrogenase 1 (MTHFD1)* gene is responsible for the generation of 10-formyl-THF, which is essential for DNA synthesis. There is a polymorphism at the nucleotide 1958 (G ® A), resulting in an substitution of alanine for glycine at the codon 653, located at the domain 10-formyl-THF synthase of the enzyme[16]. If folate availability is continuously limited, an uncontrolled repair cycle can cause frequent breaks in DNA molecule and chromosome damage, resulting in malignant cell change, contributing to cancer development[6].

Few studies investigated this polymorphism in cancer and the results are inconsistent. Kruszyna *et al.*[17] did not find any significant statistical differences in genotype and allele frequency of *MTHFD1* A1958G polymorphism in patients with larynx cancer. Matakidou *et al.*[18] and Chen *et al.*[19] did not associate the same polymorphism with lung and colorectal neoplasms, respectively. On the other hand, Li *et al.*[20] found no association of *MTHFD1* G1958A polymorphism with breast cancer.

Thus, the objectives of this study were to investigate MTHFD1 G1958A polymorphism involved in folate metabolism on head and neck cancer risk and the association between this polymorphism with risk factors (tobacco and alcohol comsumption) and clinical histopathological characteristics (primary site, lymph node involvement, and tumor extension).

Methods

This study sample consisted of 694 subjects, 240 patients with head and neck cancer (case group) and 454 subjects with no neoplasms history (control group) after obtaining the informed consent (Opinion 5566/2005, Ethics in Research Committee – CEP – Medicine School of São José do Rio Preto – FAMERP).

The patients were included in the study after a histopathological diagnosis of squamous cell carcinoma by the Service of Otorhinolaryngology and Head and Neck Surgery at *Hospital de Base*, São José do Rio Preto/SP. The tumors were classified according to the 2002 International Union of Cancer Control (IUCC) and the 2002 American Joint Committee for Cancer (AJCC) parameters into three criteria: tumor size (T), presence of regional node involvement (N) and presence of distant metastasis (M). By considering the anatomical location, they were classified as oral cavity, pharynx, larynx and unknown primary site tumors[21,22]. The blood sample DNA was obtained from the laboratory sample bank, collected from March 2000 to October 2009.

The control group consisted of 454 Brazilian blood donors without a diagnosis of cancer according to government guidelines for donated blood that is tested for 20 related diseases (http://www.hemonline.com.br/rdc153/indexframe.htm). The inclusion and exclusion criteria were, respectively, age over 40 and history of family neoplasm. Each eligible subject was interviewed to obtain data on gender, smoking habit, use of alcohol, and family history of cancer. Individuals who had smoked more than 100 cigarettes in their lifetime were considered to be tobacco consumers and individuals who drank 4 doses of alcohol per week were considered to be alcohol consumers[23,24].

Genomic DNA was obtained from peripheral blood for molecular analysis according to the modified Miller *et al.*[25] technique. Polymerase Chain Reaction – Restriction Fragment Length Polymorphism (PCR-RFLP) analysis was used to determine the *MTHFD1* G1958A polymorphism genotypes. The primers used were described by Hol *et al.*[26] (Sense: 5' – CACTCCAGTGTTTGTCCATG – 3'; Antisense: 5' – GCATCTTGAGAGCCCTGAC – 3'). Amplification was obtained by initial denaturation at 95°C (203°F) for 5 minutes, followed by 35 cycles of 30-second for DNA denaturation at 95°C (203°F), 50-second primer annealing at 53°C (127°F) and 90-second extension at 72°C (161°F). The final extension was conducted for 5 minutes at 72°C (161°F). The product of 331bp underwent enzymatic digestion by the enzyme *Msp*I for 3 hours at 37°C (98°F). Fragments of 166 bp and 70 bp were generated when the G allele was present, and the fragment with 266 bp was generated when the A allele was present.

The statistical analysis was conducted by using the software Minitab/Windows – Version 14.0 to assess the effects of the variables analyzed in head and neck cancer, and Bio Estat version 3.0 to ascertain if enotypic distributions were in Hardy-Weinberg equilibrium. The multiple logistic regression test was used to determine the effect of variables analyzed in head and neck cancer, including age (reference: < 42 years – age in quartiles), gender (reference: female), tobacco consumption status (reference: non-smokers), alcohol consumption (reference: non-alcoholics) and also to analyze clinical and histopathological variables.

The T classification was divided into small extension tumors (T1, T2) and large extension tumors (T3, T4). The N classification was dichotomized into negative node involvement (N0) and positive node involvement (N1, N2, N3). The results were shown as odds ratio (OR) and 95% confidence interval (95% CI). The significance level was set at 5% ($p \leq 0.05$). The Kaplan-Meier method was applied to evaluate the survival rate, by considering the period between the disease diagnosis and the death as end point.

Results

The results of multiple logistic regression test between groups showed significant differences between cases and controls regarding to tobacco consumption and age over 42 years ($p < 0.05$) and, therefore, were predictors of the disease (Table1).

The Hardy-Weinberg test showed the genotypic distribution was in equilibrium in the study sample (case: $c^2 = 0.7096$; $p = 0.3996$; control: $c^2 = 0.0707$; $p = 0.7903$). *MTHFD1 G1958A* polymorphism was not associated with the disease risk. The genotypic frequencies *MTHGD1* 1958GG, GA and AA were 35.83%, 45.83% and 18.34%, respectively, for the cases and 35.46%, 48.68% and 15.86%, respectively, for controls. The variant MTHFD1 1958G allele frequencies were 0.59 among the cases and 0.6 among the controls, while the MTHFD1 1958A allele frequencies were 0.41 and 0.4 among cases and controls, respectively.

The multiple logistic regression test results for the interaction between risk factors and *MTHFD1* G1958A polymorphism showed that tobacco consumption (OR: 1.68; 95% CI: 1.01-2.78; $p = 0.46$) and alcohol consumption(OR: 1.83; 95% CI: 1.06-3.15; $p = 0.03$) associated with *MTHFD1* 1958GA or AA genotype raised the risk of development of head and neck cancer (Table 2).

Regarding clinical and histopathological parameters, the results of the multiple logistic regression test showed association of polymorphism with tumor staging, being MTHFD1 1958GA or AA genotypes more frequent in stage 3 and 4 cases ($p = 0.04$) (Table3).

The overall survival rate obtained by Kaplan-Meier estimates, was 82.57 months for patients with *MTHFD1* 1958GG genotype and 59.03 for patients with *MTHFD1* 1958GA or AA genotype, as shown in Figure 1 ($p = 0.031$).

Discussion

The results showed that smoking and age over 42 years were predictors for head and neck cancer, corroborating to literature data, confirming this neoplasia is more frequent from the fourth decade of life[27] and in smokers[28-32].

Folate acts as a coenzyme in several cell reactions, being required in cell division because of its role in purine and pyrimidine biosynthesis and consequently in DNA and RNA formation[33].

The *MTHFD1* gene is involved in folate metabolism and codes for a cytosolic protein comprising 5,10-methylene-THF dehydrogenase, 5,10-methenyl-THF cyclohydrolase and 10-formyl-THS synthase. The enzymes methylene-THF dehydrogenase and methenyl-THF cyclohydrogenase, located at the same protein domain, catalyze the oxidation of 5,10-methylene-THF to 5,10-methenyl-THF, converted to 10-formyl-THF.

Table 1 – Demographic distribution, risk factors, genotypes and odds ratio (OR) for head and neck cancer

Variables	Case group (%)	Control group (%)	OR (95%CI)	p value
Tobacco consumption				
Non-smokers	41 (17.08)	267 (58.81)	Reference	Reference
Smokers	199 (82.92)	187 (41.19)	3.90 (2.46-6.20)	$p < 0.05$
Alcohol consumption				
Non-alcoholics	67 (27.92)	230 (50.66)	Reference	Reference
Alcoholics	173 (72.08)	224 (49.34)	1.56 (0.99-2.48)	$p = 0.056$
Gender				
Female	29 (12.08)	129 (28.41)	Reference	Reference
Male	211 (87.92)	325 (71.59)	1.65 (0.95-2.86)	$p = 0.073$
Age				
<42 years	8 (3.33)	177 (38.99)	Reference	Reference
42-51 years	49 (20.42)	170 (37.44)	5.22 (2.53-10.77)	$p < 0.05$
52-63 years	99 (41.25)	51 (11.23)	28.75 (13.51-61.18)	$p < 0.05$
>64 years	84 (35)	56 (12.34)	24.51 (11.57-51.92)	$p < 0.05$
MTHFD1 G1958A genotype				
GG	86 (35.83)	161 (35.46)	Reference	Reference
GA	110 (45.84)	221 (48.68)	1.38 (0.91-2.10)	$p = 0.135$
AA	44 (18.33)	72 (15.86)		

Table 2 – Distribution of risk factors related to head and neck cancer and MTHFD1 G1958A polymorphism

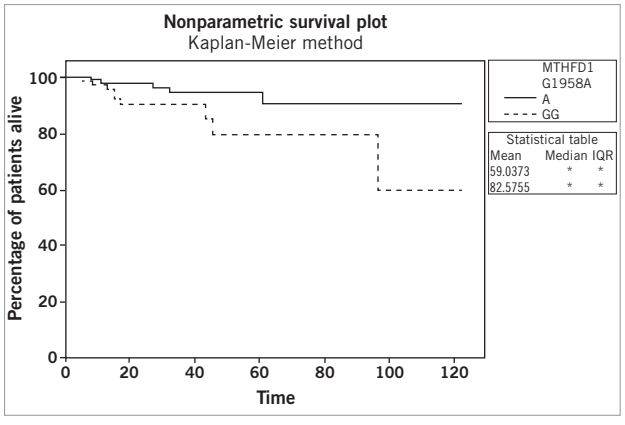
Variables	GG genotype cases/control	OR (95% CI)	GA and AA genotypes cases/control	OR (95% CI)*	p value
Age					
< 42 years	4/53	1.00 (ref)	6/123	0.38 (0.09-1.50)	p = 0.166
42-51 years	19/64	1.00 (ref)	36/105	1.78 (0.83-3.80)	p = 0.136
52-63 years	31/19	1.00 (ref)	60/32	2.02 (0.86-4.79)	p = 0.108
> 64 years	30/22	1.00 (ref)	54/34	1.31 (0.60-2.83)	p = 0.496
Gender					
Female	10/42	1.00 (ref)	19/87	1.41(0.50-3.96)	p = 0.519
Male	76/135	1.00 (ref)	119/206	1.37 (0.85-2.19)	p = 0.192
Smoking consumption					
No	23/97	1.00 (ref)	18/170	0.98 (0.45-2.14)	p = 0.964
Yes	63/64	1.00 (ref)	136/123	1.68 (1.01-2.78)	p = 0.046
Alcohol consumption					
No	29/77	1.00 (ref)	38/153	0.95 (0.49-1.85)	p = 0.890
Yes	57/84	1.00 (ref)	116/140	1.83 (1.06-3.15)	p = 0.030

Table 3 – Distribution of clinical and histopathological parameters and MTHFD1 G1958A polymorphism

Clinical parameters	GG genotype cases (%)	OR 95% CI)	GA and AA genotypes cases (%)	OR (95% CI)*	p value
Primary Site					
Oral cavity	35 (14.58)	1.00 (ref)	61 (25.42)	0.88 (0.51-1.53)	p = 0.659
Pharynx	15 (6.25)	1.00 (ref)	36 (15)	1.42 (0.72-2.81)	p = 0.312
Larynx	28 (11.67)	1.00 (ref)	45 (18.75)	0.80 (0.45-1.43)	p = 0.454
Tumor size					
T1/T2	47 (19.58)	1.00 (ref)	107 (44.58)	1.00 (ref)	
T3/T4	37 (15.42)	1.00 (ref)	49 (20.42)	0.57 (0.32-0.98)	p = 0.044
Lymph node involvement					
No	58 (24.17)	1.00 (ref)	111 (46.25)	1.00 (ref)	
Yes	26 (10.83)	1.00 (ref)	45 (18.75)	0.90 (0.50-1.62)	p = 0.721

These three sequential reactions are involved in the interconversion of THF carbon-1 derivatives, which are substrates for methionine, thymidylate and purine synthesis[19,34]. The G1950A polymorphism in this gene can be associated with cancer due to DNA synthesis changes and consequent lack of cell controll[20].

Figure 1 – Nonparametric survival plot (Kaplan-Meier) of patients with head and neck squamous cell carcinoma.



In the current study, a balanced genotypic distribution was observed, corroborating to Kruszyna *et al.*[17] study, which did not find significant statistical differences in *MTHFD1* A1958G polymorphism genotype and allele frequency.

In our study, *MTHFD1* G1958A polymorphism was not associated with head and neck cancer risk, similarly to Kruszyna *et al.*[17] findings in 131 patients with larynx cancer and 250 control patients, Matakidou *et al.*[18] findings in 619 patients with lung cancer, and Chen *et al.*[19] findings in 274 patients with colorectal cancer and 461 controls.

However, Li *et al.*[20], investigating 227 patients, showed *MTHFD1* 1958AA polymorphic genotype occurred more often in patients with breast cancer than *MTHFD1* 1958GG wild genotype. In the same study, the association between higher methylation frequency in patients with breast cancer and *MTHFD1* 1958AA polymorphic genotype was found.

In our study, there was a significant association between MTHFD1 1958GA or AA genotypes and tobacco and alcohol consumption, suggesting that individuals with these habits and GA or AA genotypes have a higher incidence of head and neck cancer. No available literature data prove this association.

The analysis of clinical and histopathological parameters confirmed T3 and T4 tumors (advanced tumors) had a higher frequency in patients with GA or AA genotypes. The study by Kruszyna *et al.*[17], analyzing the genotype significance for tumor characteristics, showed a weak association between *MTHFD1* genotypes and the tumor size.

The overall survival rate obtained by Kaplan-Meier estimate, showed the patients with *MTHFD1* 1958GG wild genotype had a higher mean survival compared to patients with *MTHFD1* 1958GA or AA genotypes (at least one polymorphic allele), confirming an association between the polymorphic allele presence and the reduced mean survival time. According to a bibliographic survey, this is the first study investigating the association between survival time and the polymorphism presence.

Conclusion

Smoking and age over 42 years modulate head and neck cancer, regardless the genetic variable. The presence of *MTHFD1* G1958A polymorphism associated with tobacco and alcohol consumption increases of head and neck cancer risk. The polymorphism is more frequent in more advanced stage tumors and in patients with a poorer prognosis. It is important to corroborate by studies the influence of *MTHFD1* gene polymorphism, as well as the influence of polymorphisms in other genes involved in folate metabolism on head and neck cancer genesis, so that the etiology and the significant correlations with clinical and histopathological characteristics of these tumors can be determined.

References

1. Argiris A, Karamouzis MV, Raben D, Ferris RL. Head and neck cancer. Lancet 2008;371:1695-709.
2. INCA. Instituto Nacional de Câncer. Avaliable from: http://www.inca.gov.br. 2010.
3. Kane, MA. The role of folates in squamous cell carcinoma of the head and neck. Cancer Detect Prev. 2006;29:46-53.
4. Lo AK, Lo KW, Tsao SW, Wong HL, Hui JW, To KF *et al.* Epstein-Barr virus infection alters cellular signal cascades in human nasopharyngeal epithelial cells. Neoplasia 2006;3:173-80.
5. Hennessey PT, Westra WH, Califano JA. Human papillomavirus and head and neck squamous cell carcinoma: recent evidence and clinical implications. Dent Res. 2009;88:300-6.
6. Linhart HG, Troen A, Bell GW, Cantu E, Chao W, Moran E *et al.* Folate deficiency induces genomic uracil misincorporation and hypomethylation but does not increase DNA point mutations. Gastroenterology 2009;136:227-35.
7. Hsiung DT, Marsit CJ, Houseman EA, Eddy K, Furniss CS, McClean MD *et al.* Global DNA methylation level in whole blood as a biomarker in head and neck squamous cell carcinoma. Cancer Epidemiol Biomarkers Prev. 2007;16:108-14.
8. Mu LN, Cao W, Zhang ZF, Yu SZ, Jiang QW, You NC, *et al.* Polymorphisms of 5,10-methylenetetralydrofolate reductase (MTHFR), fruit and vegetable intake, and the risk of stomach. Cancer Biomark. 2007;12:61-75.
9. Ouerhani S, Oliveira E, Marrakchi R, Ben Slama MR, Sfaxi M, Ayed M *et al.* Methylenetetrahydrofolate reductase and methionine synthase polymorphisms and risk of bladder cancer in a Tunisian population. Cancer Genet Cytogenet. 2007;176:48-53.
10. Pande M, Chen J, Amos CI, Lynch PM, Broaddus R, Frazier ML. Influence of methylenetetrahydrofolate reductase gene polymorphisms C677T and A1298C on age-associated risk for colorectal cancer in a caucasian lynch syndrome Population. Cancer Epidemiol Biomarkers Prev. 2007;16:1753-9.
11. Xu X, Gammon MD, Wetmur JG, Rao M, Gaudet MM, Teitelbaum SL *et al.* A functional 19-base pair deletion polymorphism of dihydrofolate reductase (DHFR) and risk of breast cancer in multivitamin users. Am J Clin Nutr. 2007;85:1098-102.
12. Kalmbach RD, Choumenkovitch SF, Troen AP, Jacques PF, D'Agostino R, Selhub J. A 19-Base pair deletion polymorphism in dihydrofolate reductase is associated with increased unmetabolized folic acid in plasma and decreased red blood cell folate. J Nutr. 2008;138:2323-7.
13. Ott N, Geddert H, Sarbia M. Polymorphisms in methionine synthase (A2756G) and cystathionine beta-synthase (844ins68) and susceptibility to carcinomas of the upper gastrointestinal tract. J Cancer Res Clin Oncol. 2008;134:405-10.
14. Garcia-Crespo D, Knock E, Jabado N, Rozen R. Intestinal neoplasia induced by low dietary folate is associated with altered tumor expression profiles and decreased apoptosis in mouse normal intestine. J Nutr. 2009;139:488-95.
15. Langevin SM, Lin D, Matsuo K, Gao CM, Takezaki T, Stolzenberg-Solomon RZ *et al.* Review and pooled analysis of studies on MTHFR C677T polymorphism and esophageal câncer. Toxicol Lett. 2009;184:73-80.
16. Krajinovic M, Lemieux-Blanchard E, Chiasson S, Primeau M, Costea I, Moghrabi A. Role of polymorphisms in MTHFR and MTHFD1 genes in the outcome of childhood acute lymphoblastic leukemia. Pharmacogenomics J. 2004;4:66-72.
17. Kruszyna L, Lianeri M, Rydzanicz M, Gajecka M, Szyfter K, Jagodzinski PP. Polymorphic variants of folate metabolism genes and the risk of laryngeal cancer. Mol Biol Rep. 2010;37:241-7.
18. Matakidou A, Galta R, Rudd MF, Webb EL, Bridle H, Eisen T *et al.* GELCAPS Consortium. Prognostic significance of folate metabolism polymorphisms for lung cancer. Br J Cancer 2007;97:247-52.
19. Chen J, Kyte C, Valcin M, Chan W, Wetmur JG, Selhub J *et al.* Polymorphisms in the one-carbon metabolic pathway, plasma folate levels and colorectal cancer in a prospective study. J Cancer 2004;110:617-20.
20. Li SY, Rong M, Iacopetta B. Germ-line variants in methyl-group metabolism genes and susceptibility to DNA methylation in human breast cancer. Oncol Rep. 2006;15:221-5.
21. Sobin LH, Wittelind CH. International union against cancer: TNM classification of malignant tumours. 6th ed. New York: Wiley; 2000.
22. Lee KJ. Essential otolaryngology-head & neck surgery. 8nd ed. New York: McGraw-Hill; 2003.
23. Kjaerhein K, Gaard M, Andersen A. The role of alcohol, tobacco, and dietary factors in upper aerogastric tract cancer: a prospective study of 10.900 Norwegian men. Cancer Causes Control. 1998;9:99-108.
24. Ahrendt SA, Chown JT, Yang SC, Wu L, Zhang MJ, Jen J *et al.* Alcohol comsuption and cigarette smoking increase the frequency of p53 mutations in nomsmall cell lung cancer. Cancer Res. 2000;31:55-9.
25. Miller SA, Dikes DD e Polesky HF. A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic Acids Res. 1988;16:1215.
26. Hol FA, Van der Put NM, Geurds MP, Heil SG, Trijbels FJ, Hamel BC *et al.* Molecular genetic analysis of the gene encoding the trifunctional enzyme MTHFD (methylenetetrahydrofolate- dehydrogenase, methenyltetrahydrofolate-cyclohydrolase, formyltetrahydrofolate synthetase) in patients with neural tube defects. Clin Genet. 1998;2:119-25.
27. Werbrouck J, De Ruyck K, Duprez F, Van Eijkeren M, Rietzschel E, Bekaert S *et al.* Single-nucleotide polymorphisms in DNA double-strand break repair genes: Association with head and neck cancer and interaction with tobacco use and alcohol consumption. Mutat Res. 2008;.656:74-81.
28. Psyrri A, DiMaio D. Human papillomavirus in cervical and head-and-neck cancer. Nat Clin Pract Oncol. 2006;5:24-31.

29. Guha N, Boffetta P, Wünsch Filho V, Eluf Neto J, Shangina O, Zaridze D *et al*. Oral health and risk of squamous cell carcinoma of the head and neck and esophagus: results of two multicentric case-control studies. Am J Epidemiol. 2007;166:1159-73.
30. Serefoglou Z, Yapijakis C, Nkenke E, Vairaktaris E. Genetic association of cytokine DNA polymorphisms with head and neck cancer. Oral Oncol. 2008;44:1093-9.
31. Yadav SS, Ruwali M, Shah PP, Mathur N, Singh RL, Pant MC *et al.* Association of poor metabolizers of cytochrome P450 2C19 with head and neck cancer and poor treatment response. Mutat Res. 2008;644:31-7.
32. Leme CVD, Raposo LS, Ruiz MT, Biselli JM, Galbiatti ALS, Maniglia JV *et al.* GSTM1 and GSTT1 genes analysis in head and neck cancer. Rev Assoc Med Bras. 2010;56:299-303.
33. Krishnaswamy K, Nair KM, Importance of folate in human nutrition. Br J Nutr. 2001;85:S115-S24.
34. Stevens VL, Mc Cullough ML, Pavluck AL, Talbot JT, Feigelson HS, Thun MJ *et al*. Association of polymorphisms in one-carbon metabolism genes and postmenopausal breast cancer incidence. Cancer Epidemiol Biomarkers 2007;16:1140-7.