The effect of cyclin D1 (CCND1) G870A-polymorphism on breast cancer risk is modified by oxidative stress among Chinese women in Singapore

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Cyclin D1 (*CCND1*), an intracellular cell-cycle regulatory protein with checkpoint function, can promote cell proliferation or induce growth arrest and apoptosis depending on the cellular context. We hypothesized that the direction of the association between the (*CCND1*) G870A-polymorphism and breast cancer risk may be modified by dietary and genetic factors influencing the oxidant–antioxidant balance, such as a dietary pattern with a high intake of *n*-6 fatty acids and a low intake of *n*-3 fatty acids, or a genetic profile that is deficient in glutathione *S*-transferases. We tested our hypothesis in a case–control study nested into the Singapore Chinese Health Study, a prospective investigation of diet and cancer in 63 000 Chinese men and women. Genomic DNA collected from 258 incident cases of breast cancer and 670 female cohort controls was examined for *CCND1, GSTM1, GSTT1* and *GSTP1* genes using fluorogenic 5′-nuclease assay. Unconditional logistic regression models were used to assess the effects with adjustment for potential confounders. All statistical tests were two-sided. The heterozygous *CCND1 GA* genotype significantly reduced the breast cancer risk in all subjects (OR = 0.67, 95% CI 0.45–0.99) when compared with the *GG* genotype. The association was restricted to women with a high (above median value) intake level of *n*-6 fatty acids (OR = 0.51, 95% CI 0.30–0.87), a low (below median value) intake level of the antagonistic marine *n*-3 fatty acids (OR = 0.54, 95% CI 0.32–0.93) or a total lack of the antioxidative *GSTM1* (OR = 0.44, 95% CI 0.25–0.80) or *GSTT1* genes (OR = 0.46, 95% CI 0.24–0.87). The effects were consistently stronger in cases with advanced disease. The *AA* genotype did not affect breast cancer risk. The results of this study are compatible with the hypothesis that the oxidant–antioxidant balance in cells is an important determinant of the direction of the cyclin D1 effect, leading either to cell proliferation or cell death.

Abbreviations: BMI, body mass index; *CCND1*, Cyclin D1 gene; CI, confidence interval; GST, glutathione *S*-transferase; OR, odds ratio; ROS, reactive oxygen species; Th1, T-helper 1.

Introduction

Cyclin D1, a protein encoded by the *CCND1* gene located on chromosome 11q13, is a key cell-cycle regulatory protein modulating the restriction point early in the G_1-phase [reviews in (1–4)]. Cyclin D1 gene (*CCND1*) is amplified or over-expressed in a variety of tumours (2,5,6). In up to 20% of breast cancers, *CCND1* is amplified and >50% of mammary tumours overexpress it (7,8). *CCND1* exhibits a common A/G polymorphism at nt 870, which modulates alternate splicing of *CCND1*. Both alleles lead to the expression of two different transcripts, but at different proportions. Several studies found the A-allele to be the major source of transcript form b, which encodes a cyclin D1 protein with an altered C-terminus. It lacks a PEST sequence postulated to target protein for rapid degradation (9–11). Carriers of one or two *A*-alleles may thus possess a longer protein half-life. Several epidemiological studies (12–17) found an increased risk for different cancer types among carriers of the *A*-allele in accordance with the cell proliferating role of cyclin D1. But the absence of an association with cancer risk or inverse associations between cancer risk or survival and the *A*-allele also were reported (18–23). Additional, modifiable risk factors were seldom taken into consideration in these prior studies.

The direction of the biological impact of cyclin D1 overexpression depends on the state of the cell in accordance with its checkpoint function. While cyclin D1 is best known for its proliferating effect (4,24,25), experimental evidence suggests that under conditions such as oxidative stress (24,26–29) or senescence (30–33), cyclin D1 can inhibit S-phase entry and DNA replication and promote growth arrest as well as apoptosis. The context-dependent dual role of cyclin D1 on cell proliferation and growth arrest, may explain the inconsistent associations observed between *CCND1* genotype and cancer risk (12,14,20,34), and emphasizes the importance of assessing the interaction between *CCND1* genotype and factors that modify the cellular micromilieu.

A potential modifier of the *CCND1* genotype/breast cancer association is oxidative stress. First, reactive oxygen species (ROS) can activate signalling cascades that ultimately lead to cell-cycle arrest and apoptosis by altering the expression of DNA damage inducible genes including *cyclin D1* and its inhibitors (35–37). Glutathione *S*-transferase (GST) enzymes which play a major role in the elimination of oxidative by-products were found to interact with *CCND1* in previous cancer susceptibility studies (38,39). The GST, GSTp, has been identified as an important regulator of JNK signalling, a member of the stress kinase family of genes involved in cell-cycle control (36,40). Second, oxidative stress from various sources has been proposed as a risk factor for breast cancer (41–43). A shift in the oxidant–antioxidant balance possibly underlies the inverse association between marine *n*-3 fatty acid intake and breast cancer risk reported by us (44), that was confined to *GSTM1, GSTT1* and *GSTP1* deficient women and

was more pronounced in patients with an advanced stage of disease (45). Kikugawa *et al.* (46), in accordance with experimental data (47–49), reported a protective effect of fish oil against oxidative stress induced DNA damage in rat liver *in vivo*, that was attributed to the lipid peroxidation products resulting from marine *n*-3 fatty acids. These lipid peroxidation metabolites are excreted less efficiently by women possessing low activity *GST* genotypes. We also found a dose-dependent increase in breast cancer risk with increasing intake of *n*-6 fatty acids among women with low intake of marine *n*-3 fatty acids (44). The tumour-enhancing effect of *n*-6 fatty acids has been related to oxidative DNA damage (47). In accordance with the impact of oxidative stress on the cell-cycle checkpoint, marine *n*-3 fatty acids inhibited (50) and *n*-6 promoted (30) *CCND1*-expression in cancer cells.

In this report, we investigated whether the association of the *CCND1* polymorphism with breast cancer risk was modified by the intake levels of *n*-3 and *n*-6 fatty acids, *GST* genotypes and stage of the disease as markers of oxidative burden.

Materials and methods

Study population

The subjects were participants of the Singapore Chinese Health Study, a population-based, prospective investigation of diet and cancer risk. The study design has been described previously (51). Briefly, from April 1993 through December 1998, we recruited 63 257 Chinese women and men from two major dialect groups in Singapore (Hokkien and Cantonese). Subjects were between the ages of 45 and 74 years and residing in government housing estates at the time of enrollment. During the period of study enrollment, 86% of the Singapore population resided in such facilities. Only women ($n = 35\ 298$) were considered in this report. At recruitment, a face-to-face interview was conducted by a trained interviewer, using a structured questionnaire to elicit information on demographics, lifetime use of tobacco, physical activity, menstrual and reproductive history, medical history and family history of cancer. The questionnaire included a dietary component assessing usual intake pattern (including frequency and portion size) during the previous 12 months on 165 food and beverage items, which were subsequently validated against a series of 24-h recalls (51). Average daily intake of *n*-6 and marine *n*-3 fatty acids, expressed as %kcal to adjust for total energy intake in the analyses, was computed for each study subject via linkage to the Singapore Food Composition Table (51). The food frequency questionnaire listed 14 seafood items commonly consumed by Chinese in Singapore, including fresh fish, fresh shellfish, dried/salted fish and canned fish. Major sources of *n*-6 fatty acids were meat (10%), grain products (20%) and cooking oils (40%) (44).

Between April 1994 and July 1999, we collected blood and single-void urine specimens from a random 3% sample of study enrollees. A 20 ml blood sample was obtained from each consenting subject and stored in a liquid nitrogen tank at $-180°$C until August 2001, when they were moved to a $-80°$C freezer for long-term storage. If the subject refused to donate blood, he/she was asked to donate buccal cells, which were collected through the use of a modified 'mouthwash' protocol based on published methods (52).

Out of 1059 female cohort participants contacted for biospecimen donation, blood ($n = 514$) and buccal cells ($n = 164$) were collected from 678 subjects, representing a participation of 64%. The control group of the present study consisted of the 670 women (203 premenopausal, 467 post-menopausal) who remained free of breast cancer as of April 30, 2002 (8 women in this subgroup of cohort subjects were first diagnosed with breast cancer between enrollment and April 30, 2002). Demographic characteristics (age, dialect group and education) of the control women who donated blood or buccal cells were comparable with all women in the cohort.

We identified incident breast cancer cases through the population-based cancer registry in Singapore (53). As of April 30, 2002, a total of 399 cases of incident breast cancer had developed among female cohort subjects. Histological and staging information on all breast cancer diagnoses were confirmed by manual review of the pathology reports and clinical charts. Blood ($n = 198$) or buccal ($n = 60$) specimens were available for 258 (65%) breast cancer cases. Of these 258 cases of breast cancer, 33 cases had *in situ* cancers, 68 had stage I, 110 had stage II, 23 had stage III and 18 had stage IV tumours, while staging information was not available for 6 cases. The 151 cases that had stage II or higher tumours (regional and metastatic disease) are classified hereafter as

having advanced disease. Breast cancer cases who did not give a blood or buccal cell sample were less educated than those who provided such a sample (44 versus 30% had no formal education). More Cantonese donated specimens (69%) compared with Hokkien (60%). The two groups were otherwise similar with respect to age at cancer diagnosis (mean age of 61 versus 59 years). In all analyses, subjects with non-informative genotypes were excluded (3 cases and 4 controls).

Informed consent forms were completed by all participants at baseline interviews and at the time of collection of biological specimens. The Institutional Review Boards at the University of Southern California and the National University of Singapore had approved this study.

Genotyping methods

DNA was purified from buffy coats of peripheral blood and buccal cell samples using a QIAamp 96 DNA Blood Kit (Qiagen, Valencia, CA). Genotyping for *CCND1*, *GSTM1*, *GSTT1* and *GSTP1* was performed using the fluorogenic 5′-nuclease assay (TaqMan Assay) (54).

The TaqMan assays were performed using a TaqMan PCR Core Reagent kit (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions.

The *CCND1* polymorphism (A/G) was previously described (14). The oligonucleotide primers for amplification of the polymorphic region of *CCND1* were GC091for (5′-CCCCAACAACTTCCTGTCCTACTA-3′) and GC091rev (5′-AGGCTGCCTGGGACATCA-3′). In addition, the fluorogenic oligonucleotide probes (TaqMan MGB Probes; ABI) used to detect each of the alleles were GC091F (5′-CCTCCTTACCGGGTCA-3′) labelled with 6-FAM to detect the *G* allele and GC091V (5′-CCTCCTTACTGGGTCA-3′) labelled with VIC to detect the *A* allele. PCR amplification using ~10 ng of genomic DNA was performed in a thermal cycler (MWG Biotech, High Point, NC) with an initial step of 95°C for 10 min followed by 50 cycles of 95°C for 25 s and 62°C for 1 min. The fluorescence profile of each well was measured in an ABI 7900HT Sequence Detection System and the results analysed with Sequence Detection Software (ABI). Experimental samples were compared with 12 controls to identify the 3 genotypes at each locus (A/A, A/G, G/G). Any samples outside the parameters defined by the controls were identified as non-informative and were retested.

The oligonucleotide primers for amplification of the polymorphic region of *GSTP1* were GC070for (5′-CCTGGTGGACATGGTGAATG-3′) and GC070rev (5′-TGCTCACACCATAGTTGGTGTAGATGA-3′). In addition, the fluorogenic MGB oligonucleotide probes used to detect each of the alleles were GC070F (5′-TGCAAATACGTCTCCCT-3′) labelled with 6-FAM and GC070V (5′-TGCAAATACATCTCCCT-3′) labelled with VIC (Applied Biosystems). PCR amplification consisted in the same procedure described above for *CCND1*.

Genotyping of the *GSTT1* and *GSTM1* loci using the TaqMan assay consisted of separate assays for *GSTT1*, *GSTM1* and the albumin control genes. The oligonucleotide primers for amplification of the *GSTT1*, *GSTM1* and albumin genes were GC003 for (5′-GTGCAAACACCTCCTGGAGAT-3′) and GC003rev (5′-AGTCCTTGGCCTTCAGAATGA-3′), GC004 for (5′-CTTGGAGGAACTCCCTGAAAAG-3′) and GC004rev (5′-TGGAACCTCCATAACACGTGA-3′), GC005 for (5′-CGATTTTCTTTTTAGGGCAGTAGC-3′) and GC005rev (5′-TGGAAACTTCTGCAAACTCAGC-3′), respectively. Fluorescent oligonucleotide probes, for detection of PCR reaction products, were synthesized to contain the dye 6-FAM (BioSearch Technologies, Novato, CA). The probes for the *GSTT1*, *GSTM1* and albumin genes were GC003FAM (5′-ATGCTGCCCATCCCTGCCC-3′), GC004FAM (5′-AAGCGGCCATGGTTTGCAGG-3′) and GC005FAM (5′-CGCCTGAGCCAGAGATTTCCCA-3′), respectively. PCR amplification using ~10 ng of genomic DNA was performed in an ABI 7900HT Sequence Detection System (Applied Biosystem) with an initial step of 95°C for 10 min followed by 50 cycles of 95°C for 25 s and 62°C for 1 min. The fluorescence profile of each well was measured in real time during the PCR amplification and the results analysed with Sequence Detection Software (ABI). Any sample with a fluorescence signal that crossed a threshold of 0.2 ΔRn before cycle 40 was considered positive for the loci analysed. Samples negative for both *GSTT1* and *GSTM1* must be positive for albumin to be called; otherwise, the sample was designated non-informative and retested. All analyses were carried out blind to case or control status.

Statistical analysis

Although we sampled our controls randomly from the whole cohort, our study is more case–control than case–cohort in design since time of follow-up was relatively short and comparable between cases and the subcohort, with only eight subjects in the latter group developing breast cancer during the observation period. We used unconditional logistic regression methods to examine the effect of the G870A-polymorphism of *CCND1* alone and together with fatty acid intake levels, and *GST* genotypes on breast cancer risk. Indicator variables for the three genotypes of *CCND1* (*GG*, *AG*, *AA*) were created using the *GG*

genotype as the reference category. The quartile distribution of marine *n*-3 and *n*-6 fatty acids (in units of %kcal) among female cohort members formed the basis for categorization of subjects. The median values of *n*-3 and *n*-6 fatty acid intake were used as cutoff points to define individuals with high (third and fourth quartiles) and low (first and second quartiles) intake levels (0.19 and 4.34 %kcal, respectively). *GSTM1* and *GSTT1 null* genotypes were subjects homozygous for the respective gene deletions. *GSTP1 AA* genotype was considered the high-activity genotype compared with the *AB/BB* genotypes. The strength of the gene–cancer associations was measured by odds ratio (OR) and its 95% confidence interval (CI). Age at recruitment (years), year of recruitment (1993–1998), dialect group (Hokkien, Cantonese), level of education (no formal education, primary school only, secondary school or higher), number of livebirths (0, 1–2, 3–4 or 5+), and age when period became regular (≤ 12, 13–14, 15–16, 17+ or never regular) were included in all models as covariates. Addition of a term for body mass index (BMI) (< 20, 20 to < 24, 24 to < 28 or ≥ 28 kg/m^2) did not appreciably alter the results and thus, was not retained in the final models. Polytomous logistic regression models (55) were used to compare cases, stratified by the stage of disease, with all controls.

Statistical analysis was carried out using the SAS version 9.0 (SAS Institute, Cary, NC). All *P*-values are two-sided and $P < 0.05$ were considered statistically significant.

Results

The characteristics of the study population are summarized in Table I. Included in the study were 670 women without and 258 women with breast cancer. Consistent with the present understanding of breast cancer risk, factors positively associated with risk were the level of education and age at first birth; characteristics inversely associated with risk were the number of livebirths, age when period became regular and age at menopause (56). Mean age at recruitment was similar for case and control groups [55.6 years (SD 7.4) and 55.8 (SD 8.0), respectively].

The distribution of *CCND1* genotype by case–control status and disease stage is summarized in Table II. The frequency of the A-allele in control women was 0.58. The genotype distribution among controls was in Hardy–Weinberg equilibrium ($P = 0.47$). Women carrying the heterozygous *GA* genotype showed an overall reduced risk of breast cancer (OR = 0.67, 95% CI = 0.45–0.99), while women carrying the *AA* genotype exhibited an OR of 0.93 (95% CI = 0.63–1.38). Restricting the analysis to patients with advanced stage disease accentuated the protective effect of the *GA* genotype (OR = 0.52, 95% CI = 0.32–0.84). Menopausal status did not modify the association between *CCND1* genotype and breast cancer (data not shown).

Table III shows the results of the *CCND1* genotype effects on breast cancer risk stratified by the intake level of marine *n*-3 and *n*-6 fatty acids. The association between the *GA* genotype and breast cancer protection was confined to women with low intake of *n*-3 fats or high intake of *n*-6 fats. The strongest association between *GA* genotype and risk reduction was found in women with both low *n*-3 and high *n*-6 fats intake (OR = 0.33, 95% CI = 0.15–0.73). The protective effects of the *GA* genotype were consistently stronger in patients with advanced disease. The power for assessment of the *GA* genotype effect among advanced stage patients with both low *n*-3 and high *n*-6 fat intake was insufficient.

Table IV presents the effects of *CCND1* genotype on breast cancer risk according to subjects' specific *GST* genotypes. The protective effect of the *CCND1-GA* genotype on breast cancer risk when compared with *GG*-genotype was restricted to women possessing either the *GSTM1-null* or *GSTT1-null* genotypes (OR 0.44, 95% CI 0.25–0.80 and 0.46, 0.24–0.87, respectively). Again, these protective effects were stronger and

Table I. Distribution of selected variables in female breast cancer patients and controls in Singapore Chinese women

	Cases (*n* = 258) *n* (%)	Controls (*n* = 670) *n* (%)
Mean age at recruitment in years ($\pm$SD)	55.6 ($\pm$7.4)	55.8 ($\pm$8.0)
Dialect group		
Cantonese	136 (52.7)	336 (50.2)
Hokkien	122 (47.3)	334 (49.8)
Education		
No formal education	77 (29.8)	257 (38.4)
Primary school	108 (41.9)	250 (37.3)
Secondary school or higher	73 (28.3)	163 (24.3)
BMI (kg/cm^2) (postmenopausal women only)[a]		
< 20	16 (8.9)	56 (12.0)
20 to < 24	104 (57.8)	272 (58.2)
24 to < 28	41 (22.8)	105 (22.5)
Age (years) when period became regular		
≤ 12	43 (16.7)	91 (13.5)
13–14	106 (41.1)	239 (35.7)
15–16	73 (28.3)	200 (29.9)
17+ or never regular	36 (13.9)	140 (20.9)
Number of live births		
None	26 (10.1)	48 (7.2)
1–2	95 (36.8)	185 (27.6)
3–4	91 (35.3)	266 (39.7)
5+	46 (17.8)	171 (25.5)
Age (years) at first live birth[b]		
≤ 20	31 (12.1)	124 (18.5)
21–25	89 (34.6)	250 (37.4)
26–30	73 (28.4)	185 (27.7)
31+	38 (14.8)	62 (9.3)
Nulliparous	26 (10.1)	48 (7.1)
Age (years) at menopause (women age 55 years or older only)[c]		
≤ 49	37 (27.0)	123 (36.6)
50–54	76 (55.5)	183 (54.5)
55+	20 (17.5)	28 (8.9)
Use of replacement hormone		
Never users	237 (91.9)	631 (94.2)
Former users	5 (1.9)	8 (1.2)
Current users	16 (6.2)	31 (4.6)
Family history of breast cancer		
No	252 (97.7)	662 (98.8)
Yes	6 (2.3)	8 (1.2)
Mean intake level of marine n-3 fatty acids in %kcal ($\pm$SD)	0.19 ($\pm$0.09)	0.20 ($\pm$0.09)
Mean intake level of n-6 fatty acids in %kcal ($\pm$SD)	4.84 ($\pm$1.84)	4.85 ($\pm$1.84)
GSTM1 genotype		
Positive	137 (53.5)	369 (55.3)
Null–Null	119 (46.5)	298 (44.7)
GSTT1 genotype		
Positive	169 (66.0)	385 (57.7)
Null–Null	87 (34.0)	282 (42.3)
GSTP1 genotype		
AA	161 (62.7)	442 (66.2)
AB	87 (33.8)	199 (29.8)
BB	9 (3.5)	27 (4.0)

The Singapore Chinese Health Study.
[a]Includes 180 cases and 467 controls who had no menstrual periods at baseline interviews.
[b]Information missing for 1 case and 1 control.
[c]Includes 137 cases and 336 controls whose age at recruitment was 55 years or older only; all premenopausal women interviewed at age 55 or older were classified into the 55+ group.

Table II. Distribution of cyclin D1 (*CCND1*) genotype in breast cancer patients and controls overall and according to stage of disease in Singapore Chinese women

CCND1 G870A genotype	Cases (n = 258) n (%)	Controls (n = 670) n (%)	OR[a] (95% CI)
Total subjects[b]			
G/G	57 (22.4)	124 (18.6)	1.00 Reference
G/A	95 (37.2)	309 (46.4)	0.67 (0.45–0.99)
A/A	103 (40.4)	233 (35.0)	0.93 (0.63–1.38)
Localized only (stage 0–1)[c]			
G/G	21 (21.0)	124 (18.6)	1.00 Reference
G/A	46 (46.0)	309 (46.4)	0.90 (0.51–1.58)
A/A	33 (33.0)	233 (35.0)	0.82 (0.45–1.49)
Advanced only (stage ≥2)[c]			
G/G	35 (23.5)	124 (18.6)	1.00 Reference
G/A	46 (30.9)	309 (46.4)	0.52 (0.32–0.84)
A/A	68 (45.6)	233 (35.0)	0.99 (0.62–1.58)

The Singapore Chinese Health Study.
[a]OR, odds ratio; CI, confidence interval. Adjusted for age at recruitment (years), year of recruitment (1993–1998), dialect group (Hokkien and Cantonese), education (no formal education, primary school, secondary school or higher), number of live births (0, 1–2, 3–4 or 5+) and age when period became regular (≤12, 13–14, 15–16, 17+ or never regular).
[b]Excluding three cases and four controls with missing genotype values.
[c]The sum is less than the total number of cases due to exclusion of cases with unknown stage (n = 6).

statistically significant only in subjects with an advanced stage of the disease. There was no evidence of a modification effect of the *CCND1* genotype–breast cancer association by *GSTP1* genotype.

Restricting the analyses to subjects with both low activity *GST* genotypes and low *n*-3 or high *n*-6 intake levels, resulted in sparse data and unstable OR estimates. Nonetheless, the *GA* genotype effects on breast cancer risk were uniformly stronger (data not shown). The OR for *GA* versus *GG* genotypes for subjects with low *n*-3 fat intake and *GSTM1-null*, *GSTT1-null* or *GSTP1-AB/AA* genotypes were 0.19 (95% CI 0.08–0.43), 0.29 (95% CI 0.11–0.73) and 0.56 (95% CI 0.23–1.36), respectively. The corresponding OR for subjects with high *n*-6 fats intake and *GSTM1-null*, *GSTT1-null*, or *GSTP1-AB/AA* genotypes were 0.25 (95% CI 0.11–0.56), 0.43 (95% CI 0.18–1.00) and 0.47 (95% CI 0.19–1.17), respectively.

Discussion

In the present study, we found a protective effect of the heterozygous *CCND1 GA* genotype on breast cancer risk which was restricted to situations of elevated oxidative stress characterized by high intake level of *n*-6 fatty acids, low intake level of the antagonistic marine *n*-3 fatty acids and absence in the host of the antioxidative GSTM1 and GSTT1 enzymes. The observed gene–cancer effects are more pronounced among cases with advanced disease, which is compatible with the hypothesized interaction between cyclin D1 and oxidative stress, since cancer cells constitutively produce redox products (36).

ROS are a significant endogenous and exogenous source of DNA damage and thus trigger cell-cycle arrest at different cell-cycle checkpoints including G_1 (57). Activation of p53 and cyclin dependent kinase inhibitors plays an important role

in the response of cells to oxidants (58). *In vitro* experiments of breast tumour and other tumour cells exposed to oxidative stressors demonstrate that cyclin D1 activation and overexpression is also able to activate molecular pathways resulting in cell-cycle arrest and apoptosis (24,28,29,59). According to these results, cyclin D1 modulates growth arrest and cell death in a p53-dependent way following exposure to ionizing radiation and oxidative DNA damage. Turner *et al.* (27) reported an increased radiosensitivity in breast cancer cells among individuals overexpressing cyclin D1 and provided *in vivo* evidence of cyclin D1 as a caretaker gene offering downstream protection against oxidative damage. Our findings extend this evidence to situations of more moderate oxidative burden than the one caused by ionizing radiation. If confirmed, they suggest that modulation of the biological function of cyclin D1 by dietary factors may lead to differential impact of the *CCND1* gene on breast cancer susceptibility and progression. The demonstration of an interactive effect between the *CCND1* gene and intake of marine *n*-3 and *n*-6 fatty acids, also strengthens our previously proposed hypothesis that the observed effects of marine *n*-3 and *n*-6 fatty acids on breast carcinogenesis are due to their respective impact on cellular oxidative burden (44,45).

Ours is the first study to investigate the association between the G870A *CCND1* polymorphism and breast cancer risk stratified by markers of oxidative stress. Only two other studies, a hospital-based case–control study in an Australian population (18) and a population-based case–control study in Austria (19), have analysed the impact of the same cyclin D1 polymorphism on breast cancer risk. Neither study found an association with breast cancer risk overall or following stratification by tumour characteristics, such as size, histological grade, lymph node involvement, estrogens receptor status or overall survival of patients. No covariates or effect modifiers were considered in either study. Previous studies have linked the *CCND1* A allele to increased susceptibility or clinical outcome in non-small cell lung cancer (11,60) and prostate cancer (15). However, discordant results between the *CCND1* A/G polymorphism and cancer risk have been observed for urinary bladder cancer (14,20) and colorectal cancer (16,17, 61). An increased susceptibility to squamous cell carcinoma of the head and neck was reported for individuals with the *AA* genotype (13), but two other investigations linked the *AA* genotype to a longer disease-free interval than the *GG* genotype (23,39). These published results are compatible with the notion of a differential role of the *CCND1* polymorphism on cancer risk as opposed to cancer progression, and are consistent with the current study observation of stronger *CCND1* effects in an advanced stage of the disease. A few studies have examined *GA* genotype separately from the *AA* genotype, which mainly found no significant *GA* genotype association with cancer risk (12–16). But the *CCND1 GA* genotype has been linked to differential overall and disease-free survival in patients with ovarian and colorectal cancers (62,63). Molecular heterosis, in which subjects heterozygous for a specific genetic polymorphism show stronger effects than subjects homozygous for either alleles, has been described (64). This differs from the classical notion of a gene–dose effect on cancer risk, and is intriguing in light of the checkpoint function of cyclin D1 and dual biological activity with distinct molecular pathways, leading to either cell-cycle promotion or programmed cell death (26,30,31,65,66). A one-directional, gene–dose dependent model may be applicable only when

Table III. Cyclin D1 (*CCND1*) genotype in relation to breast cancer risk stratified by marine *n*-3 and *n*-6 fatty acids intake level overall and according to stage of disease in Singapore Chinese women

Fatty acids intake level	*CCND1* G870A genotype						Adjusted OR[a] (95% CI)	
	No. of cases			No. of controls			GA versus GG	AA versus GG
	G/G	*G/A*	*A/A*	*G/G*	*G/A*	*A/A*		
Marine *n*-3 fatty acids intake								
Total subjects								
Low marine *n*-3 fats intake[b]	33	52	59	60	162	117	0.54 (0.32–0.93)	0.86 (0.50–1.47)
High marine *n*-3 fatty acids intake[c]	24	43	44	64	147	116	0.78 (0.44–1.41)	0.95 (0.52–1.73)
Localized stages only[d]								
Low marine *n*-3 fatty acids intake[b]	10	21	17	60	162	117	0.73 (0.32–1.67)	0.81 (0.35–1.90)
High marine *n*-3 fatty acids intake[c]	11	25	16	64	147	116	1.02 (0.47–2.23)	0.74 (0.32–1.72)
Advanced stages only[d]								
Low marine *n*-3 fatty acids intake[b]	22	29	41	60	162	117	0.45 (0.24–0.86)	0.90 (0.48–1.67)
High marine *n*-3 fatty acids intake[c]	13	17	27	64	147	116	0.57 (0.26–1.25)	1.08 (0.51–2.26)
n-6 fatty acids intake								
Total subjects								
Low *n*-6 fatty acids intake[b]	22	53	51	55	142	107	0.94 (0.51–1.72)	1.17 (0.63–2.16)
High *n*-6 fatty acids intake[c]	35	42	52	69	167	126	0.51 (0.30–0.87)	0.78 (0.46–1.33)
Localized stages only[d]								
Low *n*-6 fatty acids intake[b]	7	28	17	55	142	107	1.59 (0.65–3.92)	1.23 (0.47–3.17)
High *n*-6 fatty acids intake[c]	14	18	16	69	167	126	0.58 (0.27–1.25)	0.62 (0.28–1.37)
Advanced stages only[d]								
Low *n*-6 fatty acids intake[b]	14	24	33	55	142	107	0.63 (0.29–1.33)	1.18 (0.57–2.44)
High *n*-6 fatty acids intake[c]	21	22	35	69	167	126	0.43 (0.22–0.83)	0.84 (0.45–1.58)
Marine *n*-3 and *n*-6 fatty acids intake combined								
Total subjects								
Low marine *n*-3 and high *n*-6 fatty acids intake	19	18	31	30	78	54	0.33 (0.15–0.73)	0.86 (0.41–1.80)
Low marine *n*-3 and low *n*-6 fatty acids intake	14	34	28	30	84	63	0.84 (0.38–1.86)	0.87 (0.38–1.96)
High marine *n*-3 and high *n*-6 fatty acids intake	16	24	21	39	89	72	0.69 (0.32–1.49)	0.65 (0.29–1.45)
High marine *n*-3 and low *n*-6 fatty acids intake	8	19	23	25	58	44	1.07 (0.40–2.82)	1.66 (0.62–4.45)

The Singapore Chinese Health Study.
[a]Adjusted for age at recruitment (years), year of recruitment (1993–1998), dialect group (Hokkien, Cantonese), education (no formal education, primary school, secondary school or higher), number of livebirths (0, 1–2, 3–4, or 5+) and age when period became regular ($\leq$12, 13–14, 15–16, 17+ or never regular). OR, odds ratio; CI, confidence interval.
[b]Subjects in the first and second quartiles of consumption.
[c]Subjects in the third and fourth quartiles of consumption.
[d]The sum is less than the total number of cases due to exclusion of cases with unknown stage ($n = 6$).

gene products possess a singularly defined biological activity. The two transcripts expressed by the two alleles of the *CCND1* A870G polymorphism are both able to inhibit cell proliferation (10). Our results suggest that the heterozygous *CCND1* genotype provides the optimal proportion of transcript form a to transcript form b for the induction of proliferation arrest under conditions of oxidative stress. It is of interest that heterosis also has been observed for another gene with important regulatory activity. The cytokine IL-12 plays a critical role in promoting the development of T-helper 1 (Th1) cells and suppressing the release of Th2 cytokines. Heterozygotes in IL12 are associated with decreased expression relative to the two homozygous genotypes and with severe asthma in children (67). The molecular mechanism underlying heterosis are poorly understood. Comings and MacMurray (64) proposed several levels at which molecular heterosis may be operating—at the level of gene regulation or at the level of interaction between protein subunits. As a result, heterozygotes may produce just the right amount of protein for optimal biological activity, or they may possess a broader range of gene expression products allowing for functional plasticity. The latter explanation may be of special relevance with regard to the checkpoint function of cyclin D1.

This study has several strengths. Dietary assessment was conducted using a validated, semi-quantitative food frequency questionnaire. All exposure assessments occurred prior to cancer diagnosis, and therefore, could be presumed to be free of recall bias. Finally, the study population is genetically homogeneous since they are full-blooded descendents of natives from two contiguous prefectures in southern China. There is a theoretical concern for selection bias arisen from the higher availability of blood specimens among Cantonese versus Hokkien cases with breast cancer and the higher levels of education among cases with blood specimens versus those without blood specimens. We examined if *CCND1* genotype is related to dialect group or education among our cohort participants, and no associations were found. Given that dialect group and education are not confounders of the hypothesized *CCND1*–breast cancer association in our study population, the unequal distributions of these two factors between cases with and without blood specimens should not impact on the validity of our study findings.

A major limitation of our study is its low statistical power for the assessment of gene–environment and gene–gene interactions, due to the rather modest number of incident cases of breast cancer occurring within the relatively short

Table IV. Cyclin D1 (*CCND1*) genotype in relation to breast cancer risk stratified by GST genotypes overall and according to stage of disease in Singapore Chinese women

GSTs genotype	CCND1 G870A genotype						Adjusted OR[a] (95% CI)	
	No. of cases			No. of controls			GA versus GG	AA versus GG
	G/G	G/A	A/A	G/G	G/A	A/A		
GSTM1 genotype								
Total subjects								
GSTM1 positive	27	50	59	80	161	127	0.92 (0.53–1.58)	1.38 (0.80–2.38)
GSTM1 null–null	30	45	43	44	147	106	0.44 (0.25–0.80)	0.55 (0.30–1.00)
Localized stages only[b]								
GSTM1 positive	9	22	19	80	161	127	1.33 (0.58–3.08)	1.37 (0.58–3.22)
GSTM1 null–null	12	24	13	44	147	106	0.61 (0.28–1.33)	0.44 (0.18–1.04)
Advanced stages only[b]								
GSTM1 positive	18	25	39	80	161	127	0.65 (0.33–1.27)	1.33 (0.70–2.51)
GSTM1 null–null	17	21	29	44	147	106	0.35 (0.16–0.73)	0.60 (0.29–1.23)
Total subjects								
GSTT1 positive	33	65	70	75	171	137	0.87 (0.52–1.45)	1.12 (0.67–1.86)
GSTT1 null–null	24	30	32	49	137	96	0.46 (0.24–0.87)	0.69 (0.36–1.31)
Localized stages only[b]								
GSTT1 positive	10	30	23	75	171	137	1.36 (0.63–2.96)	1.24 (0.56–2.78)
GSTT1 null–null	11	16	9	49	137	96	0.53 (0.23–1.24)	0.40 (0.15–1.05)
Advanced stages only[b]								
GSTT1 positive	23	32	45	75	171	137	0.60 (0.32–1.10)	0.99 (0.55–1.79)
GSTT1 null–null	12	14	23	49	137	96	0.43 (0.18–1.00)	1.02 (0.46–2.26)
GSTP1 genotype								
Total subjects								
GSTP1 AA	36	62	62	79	195	167	0.66 (0.40–1.09)	0.75 (0.46–1.25)
GSTP1 AB/BB	21	33	41	45	114	66	0.62 (0.32–1.22)	1.38 (0.71–2.69)
Localized stages only[b]								
GSTP1 AA	12	25	17	79	195	167	0.83 (0.39–1.75)	0.64 (0.29–1.43)
GSTP1 AB/BB[c]	9	21	16	45	114	66	0.94 (0.39–2.26)	1.23 (0.49–3.10)
Advanced stages only[b]								
GSTP1 AA	24	34	45	79	195	167	0.53 (0.29–0.96)	0.80 (0.45–1.42)
GSTP1 AB/BB[c]	11	12	23	45	114	66	0.44 (0.18–1.08)	1.50 (0.65–3.45)

The Singapore Chinese Health Study.
[a]Adjusted for age at recruitment (years), year of recruitment (1993–1998), dialect group (Hokkien, Cantonese), education (no formal education, primary school, secondary school or higher), number of livebirths (0, 1–2, 3–4, or 5+) and age when period became regular ($<=$12, 13–14, 15–16, 17+ or never regular). OR, odds ratio; CI, confidence interval.
[b]The sum is less than the total number of cases due to exclusion of cases with unknown stage ($n = 6$).
[c]Putative 'low activity genotype'.

period of follow-up (an average of 6.0 ± 2.5 years per subject) of this cohort of Singapore Chinese women. However, as this long-term, prospective investigation continues to accrue incident cases of breast cancer, we will have the opportunity to revisit this hypothesis with sufficient statistical power. Our reason for publishing these findings now is because they are internally consistent and are biologically plausible. We hope the publication of this set of preliminary results will spur others to conduct confirmatory epidemiologic studies and mechanistic laboratory studies.

Our study results indicate that most genotype effects on disease risk cannot be generalized across diverse populations, since environmental factors may play important modifying roles. Our findings are consistent with the notion that the oxidant–antioxidant balance in cells is one important determinant of the direction of the cyclin D1 checkpoint function, leading to either cell proliferation or cell death.

References

1. Malumbres,M. and Barbacid,M. (2001) To cycle or not to cycle: a critical decision in cancer. *Nat. Rev. Cancer*, **1**, 222–231.
2. Ortega,S., Malumbres,M. and Barbacid,M. (2002) Cyclin D-dependent kinases, INK4 inhibitors and cancer. *Biochim. Biophys. Acta*, **1602**, 73–87.
3. Cook,S.J., Balmanno,K., Garner,A., Millar,T., Taverner,C. and Todd,D. (2000) Regulation of cell cycle re-entry by growth, survival and stress signalling pathways. *Biochem. Soc. Trans.*, **28**, 233–240.
4. Hunter,T. and Pines,J. (1994) Cyclins and cancer. II: Cyclin D and CDK inhibitors come of age. *Cell*, **79**, 573–582.
5. Barnes,D.M. and Gillett,C.E. (1998) Cyclin D1 in breast cancer. *Breast Cancer Res. Treat.*, **52**, 1–15.
6. Steeg,P.S. and Zhou,Q. (1998) Cyclins and breast cancer. *Breast Cancer Res. Treat.*, **52**, 17–28.

7. Sutherland,R.L. and Musgrove,E.A. (2002) Cyclin D1 and mammary carcinoma: new insights from transgenic mouse models. *Breast Cancer Res.*, **4**, 14–17.

8. Gillett,C., Smith,P., Gregory,W., Richards,M., Millis,R., Peters,G. and Barnes,D. (1996) Cyclin D1 and prognosis in human breast cancer. *Int. J. Cancer*, **69**, 92–99.

9. Solomon,D.A., Wang,Y., Fox,S.R., Lambeck,T.C., Giesting,S., Lan,Z., Senderowicz,A.M., Conti,C.J. and Knudsen,E.S. (2003) Cyclin D1 splice variants. Differential effects on localization, RB phosphorylation, and cellular transformation. *J. Biol. Chem.*, **278**, 30339–30347.

10. Sawa,H., Ohshima,T.A., Ukita,H., Murakami,H., Chiba,Y., Kamada,H., Hara,M. and Saito,I. (1998) Alternatively spliced forms of cyclin D1 modulate entry into the cell cycle in an inverse manner. *Oncogene*, **16**, 1701–1712.

11. Betticher,D.C., Thatcher,N., Altermatt,H.J., Hoban,P., Ryder,W.D. and Heighway,J. (1995) Alternate splicing produces a novel cyclin D1 transcript. *Oncogene*, **11**, 1005–1011.

12. Zhang,J., Li,Y., Wang,R. *et al.* (2003) Association of cyclin D1 (G870A) polymorphism with susceptibility to esophageal and gastric cardiac carcinoma in a northern Chinese population. *Int. J. Cancer*, **105**, 281–284.

13. Zheng,Y., Shen,H., Sturgis,E.M., Wang,L.E., Eicher,S.A., Strom,S.S., Frazier,M.L., Spitz,M.R. and Wei,Q. (2001) Cyclin D1 polymorphism and risk for squamous cell carcinoma of the head and neck: a case–control study. *Carcinogenesis*, **22**, 1195–1199.

14. Wang,L., Habuchi,T., Takahashi,T. *et al.* (2002) Cyclin D1 gene polymorphism is associated with an increased risk of urinary bladder cancer. *Carcinogenesis*, **23**, 257–264.

15. Wang,L., Habuchi,T., Mitsumori,K. *et al.* (2003) Increased risk of prostate cancer associated with *AA* genotype of cyclin D1 gene *A870G* polymorphism. *Int. J. Cancer*, **103**, 116–120.

16. Kong,S., Wei,Q., Amos,C.I., Lynch,P.M., Levin,B., Zong,J. and Frazier,M.L. (2001) Cyclin D1 polymorphism and increased risk of colorectal cancer at young age. *J. Natl Cancer Inst.*, **93**, 1106–1108.

17. Le Marchand,L., Seifried,A., Lum-Jones,A., Donlon,T. and Wilkens,L.R. (2003) Association of the cyclin D1 A870G polymorphism with advanced colorectal cancer. *JAMA*, **290**, 2843–2848.

18. Grieu,F., Malaney,S., Ward,R., Joseph,D. and Iacopetta,B. (2003) Lack of association between CCND1 G870A polymorphism and the risk of breast and colorectal cancers. *Anticancer Res.*, **23**, 4257–4259.

19. Krippl,P., Langsenlehner,U., Renner,W., Yazdani-Biuki,B., Wolf,G., Wascher,T.C., Paulweber,B., Weitzer,W., Leithner,A. and Samonigg,H. (2003) The 870G→A polymorphism of the cyclin D1 gene is not associated with breast cancer. *Breast Cancer Res. Treat.*, **82**, 165–168.

20. Cortessis,V.K., Siegmund,K., Xue,S., Ross,R.K. and Yu,M.C. (2003) A case–control study of cyclin D1 CCND1 870A→G polymorphism and bladder cancer. *Carcinogenesis*, **24**, 1645–1650.

21. Zhang,Y.J., Chen,S.Y., Chen,C.J. and Santella,R.M. (2002) Polymorphisms in cyclin *D1* gene and hepatocellular carcinoma. *Mol. Carcinog.*, **33**, 125–129.

22. Deng,L., Zhao,X.R., Pan,K.F., Wang,Y., Deng,X.Y., Lu,Y.Y. and Cao,Y. (2002) Cyclin D1 polymorphism and the susceptibility to NPC using DHPLC. *Sheng Wu Hua Xue Yu Sheng Wu Wu Li Xue Bao (Shanghai)*, **34**, 16–20.

23. Holley,S.L., Parkes,G., Matthias,C., Bockmuhl,U., Jahnke,V., Leder,K., Strange,R.C., Fryer,A.A. and Hoban,P.R. (2001) Cyclin D1 polymorphism and expression in patients with squamous cell carcinoma of the head and neck. *Am. J. Pathol.*, **159**, 1917–1924.

24. Pagano,M., Theodoras,A.M., Tam,S.W. and Draetta,G.F. (1994) Cyclin D1-mediated inhibition of repair and replicative DNA synthesis in human fibroblasts. *Genes Dev.*, **8**, 1627–1639.

25. Quelle,D.E., Ashmun,R.A., Shurtleff,S.A., Kato,J.Y., Bar-Sagi,D., Roussel,M.F. and Sherr,C.J. (1993) Overexpression of mouse D-type cyclins accelerates G$_1$ phase in rodent fibroblasts. *Genes Dev.*, **7**, 1559–1571.

26. Kinoshita,A., Wanibuchi,H., Imaoka,S., Ogawa,M., Masuda,C., Morimura,K., Funae,Y. and Fukushima,S. (2002) Formation of 8-hydroxydeoxyguanosine and cell-cycle arrest in the rat liver via generation of oxidative stress by phenobarbital: association with expression profiles of p21(WAF1/Cip1), cyclin D1 and Ogg1. *Carcinogenesis*, **23**, 341–349.

27. Turner,B.C., Gumbs,A.A., Carter,D., Glazer,P.M. and Haffty,B.G. (2000) Cyclin D1 expression and early breast cancer recurrence following lumpectomy and radiation. *Int. J. Radiat. Oncol. Biol. Phys.*, **47**, 1169–1176.

28. Pardo,F.S., Su,M. and Borek,C. (1996) Cyclin D1 induced apoptosis maintains the integrity of the G$_1$/S checkpoint following ionizing radiation irradiation. *Somat. Cell Mol. Genet.*, **22**, 135–144.

29. Coco Martin,J.M., Balkenende,A., Verschoor,T., Lallemand,F. and Michalides,R. (1999) Cyclin D1 overexpression enhances radiation-induced apoptosis and radiosensitivity in a breast tumor cell line. *Cancer Res.*, **59**, 1134–1140.

30. Atadja,P., Wong,H., Veillete,C. and Riabowol,K. (1995) Overexpression of cyclin D1 blocks proliferation of normal diploid fibroblasts. *Exp. Cell Res.*, **217**, 205–216.

31. Fukami,J., Anno,K., Ueda,K., Takahashi,T. and Ide,T. (1995) Enhanced expression of cyclin D1 in senescent human fibroblasts. *Mech. Ageing Dev.*, **81**, 139–157.

32. Freeman,R.S., Estus,S. and Johnson,E.M.Jr (1994) Analysis of cell cycle-related gene expression in postmitotic neurons: selective induction of Cyclin D1 during programmed cell death. *Neuron*, **12**, 343–355.

33. Lucibello,F.C., Sewing,A., Brusselbach,S., Burger,C. and Muller,R. (1993) Deregulation of cyclins D1 and E and suppression of cdk2 and cdk4 in senescent human fibroblasts. *J. Cell Sci.*, **105** (Pt 1), 123–133.

34. Yu,C., Lu,W., Tan,W., Xing,D., Liang,G., Miao,X. and Lin,D. (2003) Lack of association between CCND1 G870A polymorphism and risk of esophageal squamous cell carcinoma. *Cancer Epidemiol. Biomarkers Prev.*, **12**, 176.

35. Hershenson,M.B. (2004) p21Waf1/Cip1 and the prevention of oxidative stress. *Am. J. Physiol. Lung Cell Mol. Physiol.*, **286**, L502–L505.

36. Loo,G. (2003) Redox-sensitive mechanisms of phytochemical-mediated inhibition of cancer cell proliferation (review). *J. Nutr. Biochem.*, **14**, 64–73.

37. Pearce,A.K. and Humphrey,T.C. (2001) Integrating stress-response and cell-cycle checkpoint pathways. *Trends Cell Biol.*, **11**, 426–33.

38. Ramachandran,S., Hoban,P.R., Ichii-Jones,F., Pleasants,L., Ali-Osman,F., Lear,J.T., Smith,A.G., Bowers,B., Jones,P.W., Fryer,A.A. and Strange,R.C. (2000) Glutathione *S*-transferase *GSTP1* and cyclin D1 genotypes: association with numbers of basal cell carcinomas in a patient subgroup at high-risk of multiple tumours. *Pharmacogenetics*, **10**, 545–556.

39. Matthias,C., Jahnke,V., Jones,P.W., Hoban,P.R., Alldersea,J.E., Worrall,S.F., Fryer,A.A. and Strange,R.C. (1999) Cyclin D1, glutathione *S*-transferase, and cytochrome P450 genotypes and outcome in patients with upper aerodigestive tract cancers: assessment of the importance of individual genes using multivariate analysis. *Cancer Epidemiol. Biomarkers Prev.*, **8**, 815–823.

40. Adler,V., Yin,Z., Fuchs,S.Y. *et al.* (1999) Regulation of JNK signaling by GSTp. *EMBO J.*, **18**, 1321–1334.

41. Ambrosone,C.B. (2000) Oxidants and antioxidants in breast cancer. *Antioxid. Redox Signal*, **2**, 903–917.

42. Kang,D.H. (2002) Oxidative stress, DNA damage, and breast cancer. *AACN Clin. Issues*, **13**, 540–549.

43. Brown,N.S. and Bicknell,R. (2001) Hypoxia and oxidative stress in breast cancer. Oxidative stress: its effects on the growth, metastatic potential and response to therapy of breast cancer. *Breast Cancer Res.*, **3**, 323–327.

44. Gago-Dominguez,M., Yuan,J.M., Sun,C.L., Lee,H.P. and Yu,M.C. (2003) Opposing effects of dietary n-3 and n-6 fatty acids on mammary carcinogenesis: The Singapore Chinese Health Study. *Br. J. Cancer*, **89**, 1686–1692.

45. Gago-Dominguez,M., Castelao,J.E., Sun,C.L., Van Den Berg,D., Koh,W.P., Lee,H.P. and Yu,M.C. (2004) Marine *n*-3 fatty acid intake, glutathione *S*-transferase polymorphisms and breast cancer risk in post-menopausal Chinese women in Singapore. *Carcinogenesis*, **25**, 2143–2147.

46. Kikugawa,K., Yasuhara,Y., Ando,K., Koyama,K., Hiramoto,K. and Suzuki,M. (2003) Protective effect of supplementation of fish oil with high n-3 polyunsaturated fatty acids against oxidative stress-induced DNA damage of rat liver *in vivo*. *J. Agric. Food Chem.*, **51**, 6073–6079.

47. Bartsch,H., Nair,J. and Owen,R.W. (1999) Dietary polyunsaturated fatty acids and cancers of the breast and colorectum: emerging evidence for their role as risk modifiers. *Carcinogenesis*, **20**, 2209–2218.

48. Barbosa,D.S., Cecchini,R., El Kadri,M.Z., Rodriguez,M.A., Burini,R.C. and Dichi,I. (2003) Decreased oxidative stress in patients with ulcerative colitis supplemented with fish oil *omega*-3 fatty acids. *Nutrition*, **19**, 837–842.

49. Mori,T.A., Woodman,R.J., Burke,V., Puddey,I.B., Croft,K.D. and Beilin,L.J. (2003) Effect of eicosapentaenoic acid and docosahexaenoic

acid on oxidative stress and inflammatory markers in treated-hypertensive type 2 diabetic subjects. *Free Radic. Biol. Med.*, **35**, 772–781.

50. Palakurthi,S.S., Fluckiger,R., Aktas,H., Changolkar,A.K., Shahsafaei,A., Harneit,S., Kilic,E. and Halperin,J.A. (2000) Inhibition of translation initiation mediates the anticancer effect of the *n*-3 polyunsaturated fatty acid eicosapentaenoic acid. *Cancer Res.*, **60**, 2919–2925.

51. Hankin,J.H., Stram,D.O., Arakawa,K., Park,S., Low,S.H., Lee,H.P. and Yu,M.C. (2001) Singapore Chinese Health Study: development, validation, and calibration of the quantitative food frequency questionnaire. *Nutr. Cancer*, **39**, 187–195.

52. Lum,A. and Le Marchand,L. (1998) A simple mouthwash method for obtaining genomic DNA in molecular epidemiological studies. *Cancer Epidemiol. Biomarkers Prev.*, **7**, 719–724.

53. Chia,K.S., Lee,J.J., Wong,J.L., Gao,W., Lee,H.P. and Shanmugaratnam,K. (2002) Cancer incidence in Singapore, 1998 to 1999. *Ann. Acad. Med. Singapore*, **31**, 745–750.

54. Lee,L.G., Connell,C.R. and Bloch,W. (1993) Allelic discrimination by nick-translation PCR with fluorogenic probes. *Nucleic Acids Res.*, **21**, 3761–3766.

55. Hosmer,W. and Lemeshow,S. (1989) Polytomous logistic regression. In Hosmer,W. and Lemeshow,S. (eds) *Applied Logistic Regression*. John Wiley & Sons, New York, pp. 216–245.

56. Koh,W.P., Yuan,J.M., Sun,C.L., van den Berg,D., Seow,A., Lee,H.P. and Yu,M.C. (2003) Angiotensin I-converting enzyme (*ACE*) gene polymorphism and breast cancer risk among Chinese women in Singapore. *Cancer Res.*, **63**, 573–578.

57. Shackelford,R.E., Kaufmann,W.K. and Paules,R.S. (2000) Oxidative stress and cell cycle checkpoint function. *Free Radic. Biol. Med.*, **28**, 1387–404.

58. Clement,A., Henrion-Caude,A., Besnard,V. and Corroyer,S. (2001) Role of cyclins in epithelial response to oxidants. *Am. J. Respir. Crit. Care Med.*, **164**, S81–S84.

59. Zhou,Q., Fukushima,P., DeGraff,W., Mitchell,J.B., Stetler-Stevenson,M., Ashkenazi,A. and Steeg,P.S. (2000) Radiation and the Apo2L/TRAIL apoptotic pathway preferentially inhibit the colonization of premalignant human breast cells overexpressing cyclin D1. *Cancer Res.*, **60**, 2611–2615.

60. Qiuling,S., Yuxin,Z., Suhua,Z., Cheng,X., Shuguang,L. and Fengsheng,H. (2003) Cyclin D1 gene polymorphism and susceptibility to lung cancer in a Chinese population. *Carcinogenesis*, **24**, 1499–1503.

61. McKay,J.A., Douglas,J.J., Ross,V.G., Curran,S., Murray,G.I., Cassidy,J. and McLeod,H.L. (2000) Cyclin D1 protein expression and gene polymorphism in colorectal cancer. Aberdeen colorectal initiative. *Int. J. Cancer*, **88**, 77–81.

62. Kong,S., Amos,C.I., Luthra,R., Lynch,P.M., Levin,B. and Frazier,M.L. (2000) Effects of cyclin D1 polymorphism on age of onset of hereditary nonpolyposis colorectal cancer. *Cancer Res.*, **60**, 249–252.

63. Dhar,K.K., Branigan,K., Howells,R.E., Musgrove,C., Jones,P.W., Strange,R.C., Fryer,A.A., Redman,C.W. and Hoban,P.R. (1999) Prognostic significance of cyclin D1 gene (*CCND1*) polymorphism in epithelial ovarian cancer. *Int. J. Gynecol. Cancer*, **9**, 342–347.

64. Comings,D.E. and MacMurray,J.P. (2000) Molecular heterosis: a review. *Mol. Genet. Metab.*, **71**, 19–31.

65. Fukami-Kobayashi,J. and Mitsui,Y. (1999) Cyclin D1 inhibits cell proliferation through binding to PCNA and cdk2. *Exp. Cell Res.*, **246**, 338–347.

66. Del Sal,G., Murphy,M., Ruaro,E., Lazarevic,D., Levine,A.J. and Schneider,C. (1996) Cyclin D1 and p21/waf1 are both involved in p53 growth suppression. *Oncogene*, **12**, 177–185.

67. Morahan,G., Huang,D., Wu,M., Holt,B.J., White,G.P., Kendall,G.E., Sly,P.D. and Holt,P.G. (2002) Association of IL12B promoter polymorphism with severity of atopic and non-atopic asthma in children. *Lancet*, **360**, 455–459.