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A Comparison of Approaches for Association Studies of Polymorphisms and Colorectal Cancer Risk

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Abstract

Aim—Meta-analyses have evaluated associations between polymorphisms and colorectal cancer risk, but the quality of individual studies used to inform them may vary substantially. Our aim was to apply well-established quality-control criteria to individual association studies, and then compare the results of meta-analyses that included or excluded studies that did not meet these criteria.

Method—We used meta-analyses of studies reporting a relationship between polymorphisms and colorectal cancer published between 1996 and 2008. Polymorphism-cancer associations were derived in separate meta-analyses including only those meeting quality-control criteria.

Results—Relative odds ratios varied substantially between the open and restricted group meta-analyses for all variants except *MTHFR* 677 CT. However, the associations were modest and the direction of relative risk did not change after applying criteria. Publication bias was detected for all associations, except the restricted set of studies for *GSTP1* GG.

Conclusion—We observed variation in calculated relative risk and changes in tests for publication bias depending on inclusion criteria used for association studies of polymorphisms and colorectal cancer. Standardizing study inclusion criteria may reduce the variation in findings for meta-analyses of gene-association studies of common diseases such as colorectal cancer.

Keywords

colorectal cancer; genetic polymorphisms; meta-analysis

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*The Figure references are found in Appendix A, and are sorted alphabetically, then by year (year indicated on figure when necessary). The #s 1-21 in parentheses after the authors in the figures indicate that study's country of origin, represented in Appendix B.

Introduction

Colorectal cancer is the third most common cancer for both men and women in the United States, and the second leading cause of cancer mortality for both sexes combined [1]. Risk factors for colorectal cancer include behavioral and genetic factors. Numerous studies have identified high-prevalence, low-penetrance genetic variants that appear to be associated with higher risk of developing colorectal cancer. These studies, however, vary widely in methodology, number of subjects and selection of control groups.

A number of studies have attempted to summarize this literature using meta- or pooled analyses. An earlier meta-analysis of polymorphism studies noted that many lacked statistical power to identify effects and failed to control for potential confounding factors [2]. A later review [3] identified 16 such analyses of colorectal cancer, addressing the associations between 34 polymorphisms and cancer risk; many of these polymorphisms were analyzed in multiple studies. The results of meta-analyses of candidate polymorphisms have varied as the availability of studies has grown over time, both with regard to the significance and strength of association between the variants and the risk of developing colorectal cancer [2, 4, 5]. While these studies have focused on being comprehensive with regard to available data, less attention has been paid to the quality of the individual studies included in these meta-analyses, and the impact of including lower quality studies on the overall findings. Several recent reviews have highlighted the importance of adhering to specific standards when designing and conducting gene association studies [6-8].

We present results from a comprehensive review of studies published through to 2008 addressing multiple genetic polymorphisms and colorectal cancer risk. Our purpose was to apply well-established quality metrics to these studies, and to compare the results of meta-analyses that included or excluded studies that did not meet these metrics. We evaluated the evidence for differences in colorectal cancer risk associated with single nucleotide polymorphisms (SNPs) and deletions, and assessed the strength of reported associations with these polymorphisms when study inclusion criteria were varied.

Method

Study Selection

Studies reporting a relationship between polymorphisms and colorectal cancer incidence were identified through a Medline search employing the search term algorithm listed in Table 1. To increase the sensitivity of the search for identifying studies of colorectal cancer, we included MeSH search terms for both colorectal neoplasms and colonic polyps. Studies were limited to those published in English from 1996 to 2008 inclusive. In a second-tier search, we used the individual polymorphisms as search terms to identify any additional articles that may have been missed. This yielded two additional articles, one describing *GSTM1* and *GSTT1* and another on *NAT1*, which were subsequently included in the analysis. We also reviewed reference lists from other meta-analyses of polymorphisms associated with colorectal cancer for any studies not identified by our search strategy.

Only case-control studies estimating colorectal cancer risk from incidence data were included; those based on mortality data alone were excluded. A study was also excluded if it included the same population as a more recently published article (the more recent article was then used) or if polyps (adenomatous or not) were the primary endpoint. Additionally, if the control group did not reflect the population from which cases arose, then the study was excluded. We also excluded studies of polymorphisms that were not biallelic, such as *NAT2*; the definitions of risk-conferring genotypes in such studies may vary, making them difficult to summarize.

We subsequently evaluated the studies using criteria that reflected high study quality for genetic association studies for common variants: (1) exclusion of cases with known colorectal cancer mutations, such as Lynch syndrome, familial adenomatous polyposis, etc.; (2) genotyping methods were described; (3) studies including only histologically confirmed colon or rectal cancer cases; (4) controls were matched to cases on age and sex at a minimum; (5) researchers performing the genotyping assays and those conducting the analysis were blinded to the identity of cases and controls; (6) Hardy-Weinberg equilibrium was assessed or was assessible from the study; (7) at least 100 colorectal cancer cases were included in the analysis. While we originally intended to use all of these as inclusion criteria, requiring all seven criteria left too few articles to complete a meaningful meta-analysis analysis. Because a small number of articles met control matching, blinding, and Hardy-Weinberg equilibrium criteria, these were dropped as inclusion criteria for the more restrictive analysis. A count of the number of articles meeting each of the above criteria appears in Table 2.

Separate meta-analyses were then constructed, first for all articles and second for those meeting exclusion criteria 1-3 and 7 listed above.

Analysis

For each polymorphism and reference group, meta-analysis was used to combine odds ratios (ORs) across studies and obtain an overall measure of association. We estimated risk separately for homozygous and heterozygous minor and major alleles. Random effects models based on the DerSimonian-Laird method were used in all cases to estimate pooled ORs and 95% confidence intervals (95% CIs) [9]. The method of inverse variance weighting was used for pooling. Heterogeneity was assessed with a chi-square test statistic, with degrees of freedom equal to the number of studies less one. This test is based on the between-study variance (moment estimator of DerSimonian-Laird) [9]. Publication bias for all studies and the restricted set of studies related to each polymorphism was assessed informally by evaluating symmetry in the funnel plot, and more formally with a hypothesis test based on the rank correlation between standardized treatment estimates and the variance of estimated treatment effects, with Kendall's tau used as the correlation measure [10]. The test statistic follows a standard normal distribution.

Results

The initial search yielded a total of 211 unique citations describing studies of 303 separate polymorphisms (several articles described more than one polymorphism). We focused our analysis on the six most commonly-analyzed polymorphisms that met our inclusion criteria. Studies included populations in Australia, Brazil, China, Egypt, Germany, Hungary, India, Iran, Italy, Japan, Korea, the Netherlands, Norway, Romania, Singapore, Spain, Sweden, Taiwan, Turkey, the United Kingdom, and the United States. For studies which met our restrictive criteria, the most commonly-described variant was the *GSTM1* deletion (20 articles overall, five meeting aforementioned criteria, or 25%), followed by *MTHFR* C677T (10 articles overall, 6 [60%] meeting all criteria) and *MTHFR* A1298C (8 overall, 6 [75%] meeting all criteria) and *GSTT1* deletion; (15 overall and 3 or 20% meeting all criteria). Some studies included population-based cases and controls, while others used hospital-based cases and controls. Across polymorphisms, the percentage of studies included for meta-analysis under more restrictive criteria ranged from 11% to 75% by polymorphism. All studies described the genotyping method used. Depending on polymorphism, persons with mutations known to elevate colorectal cancer risk were excluded in 22%-75% of the evaluated studies. With the exception of those considering *NAT1*, most studies only included colorectal cancer cases that were histologically confirmed. Table 2 lists inclusion and exclusion criteria and the number of studies meeting those criteria for each

polymorphism. Table 3 summarizes odds ratio estimates and the results of our assessment of publication bias. We found evidence of such bias for variant *GSTT1*. The direction of the relative risk did not change for any variant when they were compared using the open and restricted criteria.

The meta-analyses summarizing association studies are listed in Figures 1-9 and below we report results using random effects models. Before applying study inclusion criteria, *GSTM1* and *GSTT1* null variants showed modest, though statistically significantly increased risks of colorectal cancer when using the less restrictive study inclusion criteria (OR_{GSTM1}:1.21, 95% CI: 1.09-1.33; OR_{GSTT1}: 1.32, 95% CI: 1.08-1.61, Figures 1a, 2a). When all study inclusion criteria were employed, there was no longer a statistically significant increased risk of colorectal cancer for *GSTM1*, OR_{GSTM1} (1.16, 95% CI 0.94-1.43, Figure 1b) however a significant positive association remained for *GSTT1* OR_{GSTT1} (1.30, 95% CI 1.04-1.63, Figure 2b). For *GSTP1* using either set of inclusion criteria, the homozygous variant genotype was not associated with risk (Figures 7a, 7b), but heterozygotes showed a positive and significantly increased risk (OR: 1.24, 95% CI: 1.03-1.49, Figure 8) when using less rigorous criteria. Utilizing stricter inclusion criteria, no articles met all defined criteria for the heterozygous genotype, thus this analysis was not performed.

There was no difference in risk associated with the *MTHFR* 677 CT heterozygous or TT homozygous genotypes regardless of the inclusion criteria used (Figures 3a, 3b, 4a, 4b). For *MTHFR* 1298 AC and CC variants and *NAT1* rapid acetylators, risk was not significantly different from 1.0 regardless of criteria used (Figures 5a, 5b, 6a, 6b, 9).

Discussion and Conclusions

We conducted a comparative meta-analysis of commonly-studied genetic polymorphisms and their associations with colorectal cancer risk, with the primary purpose of examining the impact of applying stricter levels of study quality on what is included in a meta-analysis, and in turn the impact on the results of those analyses. We observed variation in risk depending on inclusion criteria used for the studies in the analyses. For example, using less restrictive inclusion criteria, we observed modest increases in risk associated with the null variants *of GSTM1* and the heterozygous variant of *GSTP1*. Risk increases were observed with *GSTT1* regardless of study inclusion criteria used. Changing inclusion criteria had no substantive impact on the level or significance of risk for the homozygous variant of *GSTP1*, *MTHFR* A1298C, *MTHFR* C677T or *NAT1* variants.

Our relative risk estimate for *GSTM1* (OR=1.21) is somewhat higher than those in earlier meta-analyses, which range from 0.92 to 1.10; this increased risk was statistically significant, where estimates from previous meta-analyses were not [2-5, 11, 12]. Our findings for *GSTT1* and *GSTP1* were comparable to results from earlier meta-analyses [2-5].

Our estimated OR for *MTHFR* 677 TT (0.86) was comparable to earlier estimates comparing TT to CC genotypes (both 0.83), however we did not find the OR to be statistically significant [3, 13, 14], possibly because fewer studies were included. Our analysis did not confirm a previous finding of a significant decrease in risk associated with *MTHFR* 1298 CC [14]. However, the summary OR we estimated was similar to that estimated for *MTHFR* 677 TT, and study power may have limited our ability to obtain a more stable and statistically significant risk estimate for *MTHFR* 1298 CC. A complicating factor is that the studies generally did not estimate *MTHFR* A1298C in combination with the highly linked C677T polymorphism. Our group has previously shown that this can result

in misleading risk estimates [15]. Like ours, earlier analyses did not find significant changes in colorectal cancer risk associated with *NAT1* variants [2-5].

Although the associations between a particular polymorphism and risk for colorectal cancer with either the full or restricted sets of studies were modest, relative to each other, the ORs changed substantially for many. This variance raises the issue of whether developing and applying quality standards to improve the homogeneity of study designs would reduce inter-study variability in results. Certainly, many factors beyond study design can influence risk (e.g., unmeasured population characteristics), but an argument can be made that developing quality standards is needed to control factors that can influence results from retrospective association studies. Groups involved with improving the state of research for retrospective gene-association studies may wish to consider developing guidelines for researchers in this area.

The glutathione-S transferases (GSTs) are phase II enzymes that detoxify carcinogens including heterocyclic aromatic amines found in cooked meat and polycyclic aromatic hydrocarbons in cigarette smoke; both exposures have been associated with increased risk of colorectal cancer [2, 4]. Those with the null variants of these genes lack enzyme activity, resulting in reduced ability to detoxify carcinogens. Thus, we hypothesized that null variants in the GSTs would be associated with increased risk, as reported here for *GSTM1* and *GSTT1*. The N-acetyl transferases, including *NAT1*, are also phase II enzymes; they eliminate some carcinogens but activate others, including heterocyclic aromatic amines, so their effect on cancer risk is more difficult to predict [2]. In our meta-analysis, the *NAT1* slow acetylator variant was not associated with colorectal cancer risk.

The key regulatory enzyme in folate metabolism, 5,10 methylene-tetrahydrofolate reductase (*MTHFR*), and the variants studied here (C677T and A1298C) are associated with decreased enzyme activity and (for 677T) lower plasma folate levels. The influence of folate on colorectal carcinogenesis may be attributable to its roles in nucleotide synthesis and DNA methylation. In this analysis, *MTHFR* 677 TT was not associated with a statistically significant decrease in the risk of colorectal cancer, and *MTHFR* 1298 CC with the suggestion of a decrease in risk. However, both of these variants have been shown to be associated with risk differently under varying folate status [16]. The lack of consideration for this gene-environment interaction by default limits the value of meta-analyses for *MTHFR*.

The genetic variants studied here are expected to have modest effects on risk in the individuals who carry them. However, these variants are common in the population; for example, the homozygous null variant of *GSTM1* is found in about 50% of Caucasians [17]. As a result, even modest individual risks may have large effects on cancer risk at the population level. Studying more than one genetic polymorphism in combination may also reveal important effects. Most enzymes act not independently, but in biologic pathways. There are strong evolutionary pressures to maintain the stability of such a pathway, and it is unlikely that any single mutation will affect its function. This robustness in biologic systems implies that multiple disturbances are needed (e.g., multiple genetic polymorphisms, or evolutionary “stress” on the system) in order to alter their function.

The interaction between genetic polymorphisms and environmental exposures is also important. For example, a study of GST variants and controlled diets found that *GSTM1* genotype modified the effect of diets including specific vegetables on serum levels and activity of GST [18]. Similarly, genetic polymorphisms in folate metabolism alter risk differentially depending on folate status [19], and appear to play an even greater role under the “stress” of chemotherapeutic agents that target folate metabolism [20, 21]. One may

therefore expect that whole-genome association studies will yield many more meaningful "hits" once environmental factors are considered.

This analysis has several limitations. Some of the included studies may suffer from selection bias and some recruited controls in clinical settings rather than population-based settings. This was noted in the statistical analysis, but both types of studies were included in the overall meta-analyses. We conducted two analyses of each genetic variant, excluding studies based on our quality criteria. Some quality concerns remained with all studies that were ultimately included in the meta-analysis. Many did not mention whether the cancer was histologically confirmed. Blinding of the laboratory as to case and control status was rarely mentioned, although we expect it was commonly done. Hardy-Weinberg equilibrium was assessed in only three studies and was not calculable for more than half. Many studies failed to control for confounding by age and sex. A limitation is that the number of studies included was often fairly small, limiting statistical power for precise risk estimates. Finally, this study is mainly restricted to the analysis of GST polymorphisms and *MTHFR* polymorphisms, variants with defined functional impact. Thus, it is not necessarily representative of all known risk alleles in colorectal cancer.

Documentation of racial/ethnic origin was not considered as a strict inclusion criterion for our study. Generally, the risk associated with a particular genotype should be similar across different ethnicities independent of allele frequency, unless strong modifying alleles are present. Further, determining ethnicity can be difficult, particularly in populations with large immigrant populations. In the future, an additional quality control criterion could be to include genotyping of ethnic reference panels or restriction to the predominant race/ethnicity.

We identified publication bias for *GSTP1* heterozygotes (p=0.02) using open criteria. No publication bias was found for any polymorphism using restrictive criteria. The heterozygous genotype of *GSTP1* was significantly associated with higher colorectal cancer risk; this finding of publication bias suggests that one should interpret this association with caution, particularly its statistical significance.

This meta-analysis found that *GSTT1, GSTP1* AG, and *GSTM1* variants are associated with modest but significant increases in the risk of colorectal cancer. Our findings did not support an association between the *MTHFR* A1298C polymorphism (considered individually, without the context of the *MTHFR* C677T variant), *GSTP1* GG, *MTHFR* 677, or the *NAT1* slow acetylator polymorphism and altered risk of colorectal cancer. Considering the inherent robustness of biologic pathways, future studies need to focus increasingly on standardized evaluations of gene-gene and gene-environment interactions, and appropriate tools for meta-analyses of such interactions need to be developed.

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Appendix

Appendix A:

Figure References

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Appendix B: Figure Country List

Superscript number	Country
1	Brazil
2	China
3	Egypt
4	Germany
5	Holland
6	Hungary
7	India
8	Iran
9	Italy
10	Japan
11	Korea
12	Norway
13	Singapore
14	Spain
15	Sweden
16	Taiwan
17	Turkey
18	UK
19	US
20	Romania
21	Australia

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What is new in this paper?

While previous meta-analyses of polymorphism studies in colorectal cancer have focused on being comprehensive with regard to available data, this study addresses the quality of the individual studies included in this analysis, and the impact of including lower-quality studies on the overall findings.

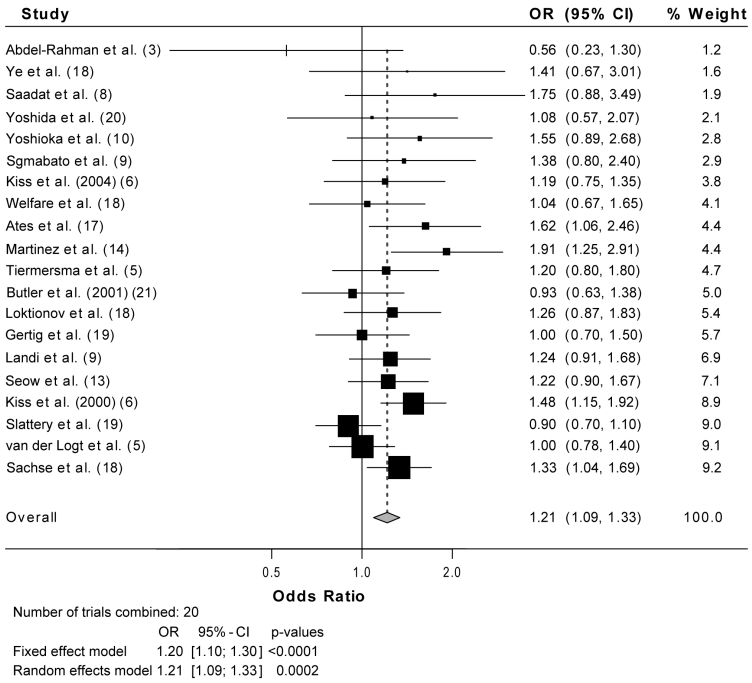

Figure 1a.
GSTM1 deletion (open criteria)

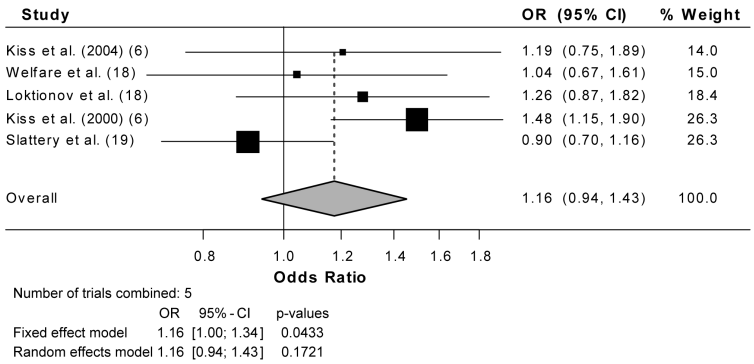

Figure 1b.
GSTM1 deletion (restricted criteria)

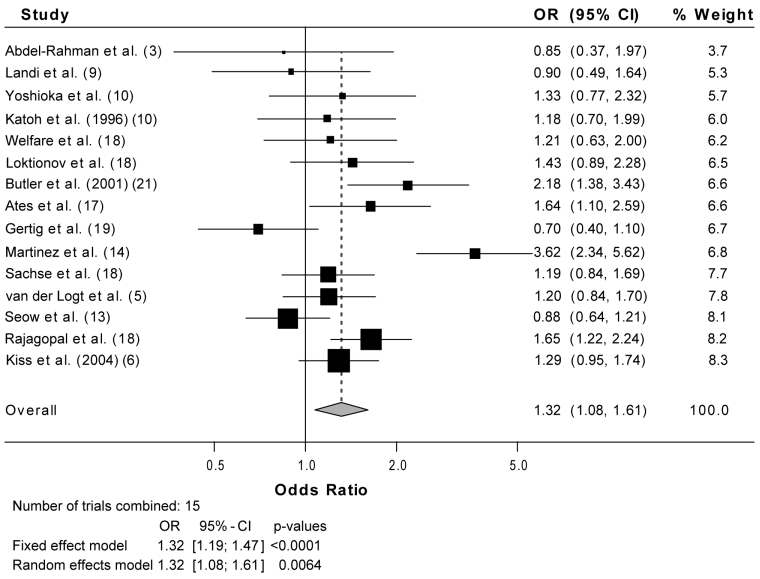

Figure 2a.
GSTT1 deletion (open criteria)

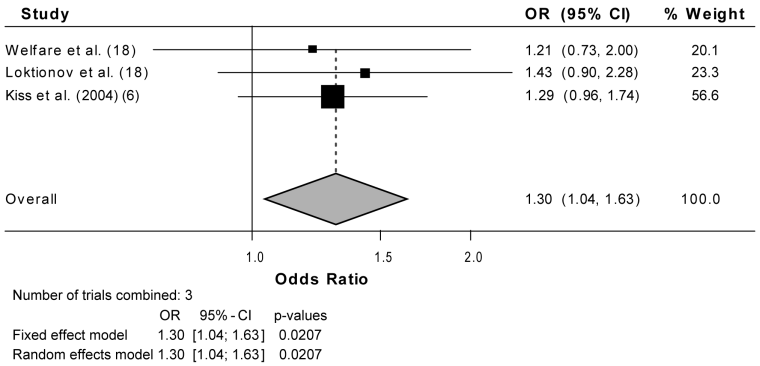

Figure 2b.
GSTT1 deletion (restricted criteria)

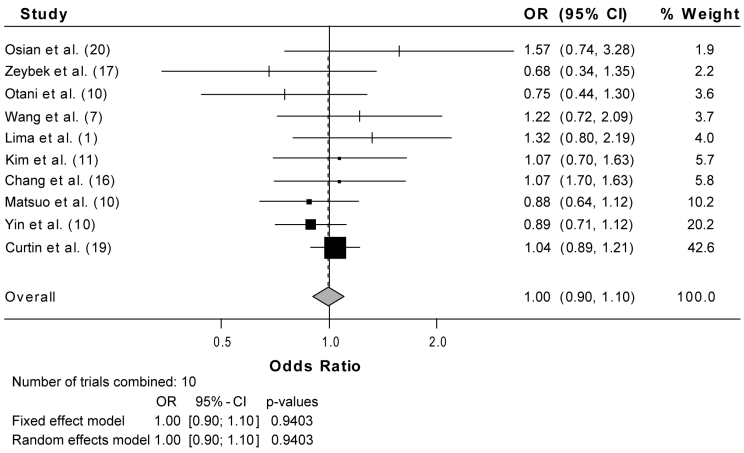

Figure 3a.
MTHFR 677 CT vs CC (open criteria)

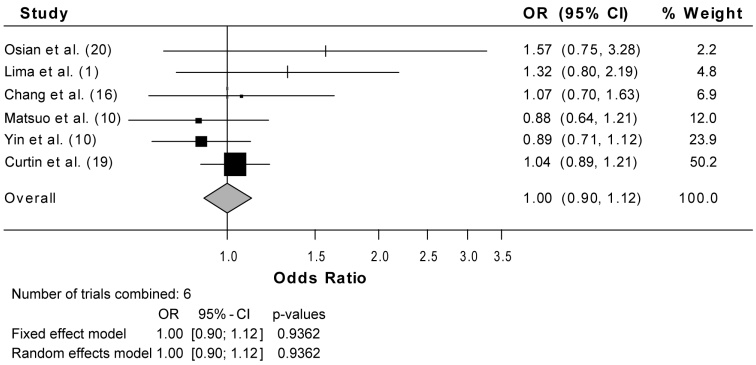

Figure 3b.
MTHFR 677 CT vs CC (restricted criteria)

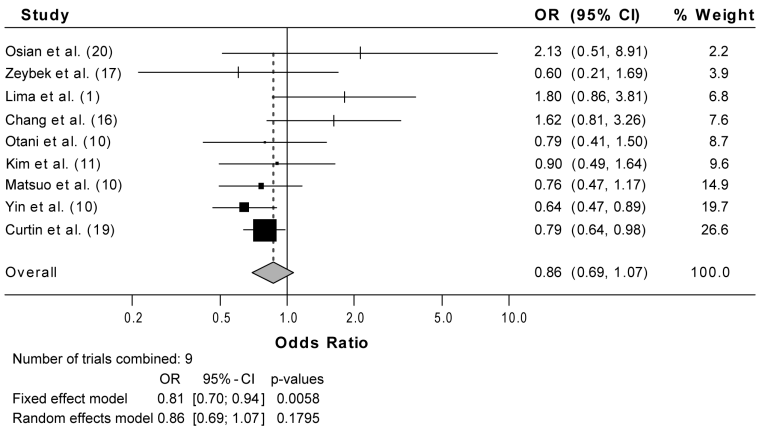

Figure 4a.
MTHFR 677 TT vs CC (open criteria)

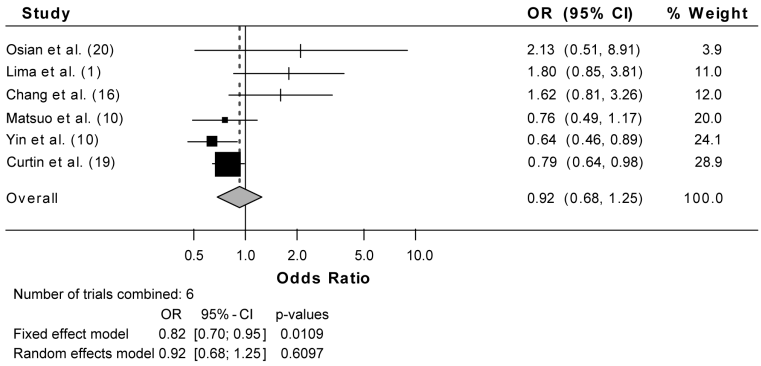

Figure 4b.
MTHFR 677 TT vs CC (restricted criteria)

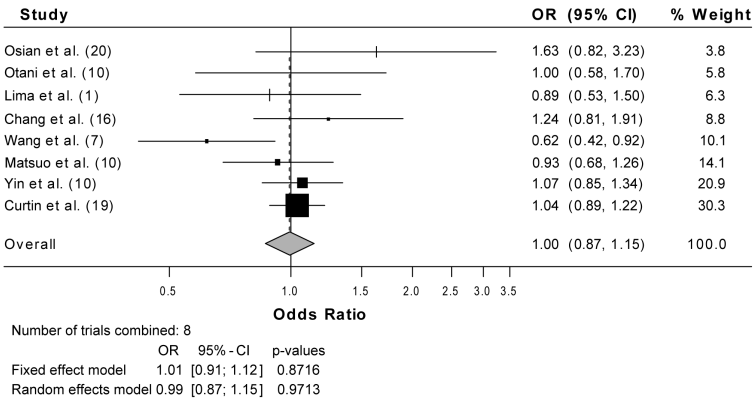

Figure 5a.
MTHFR 1298 AC vs AA (open criteria)

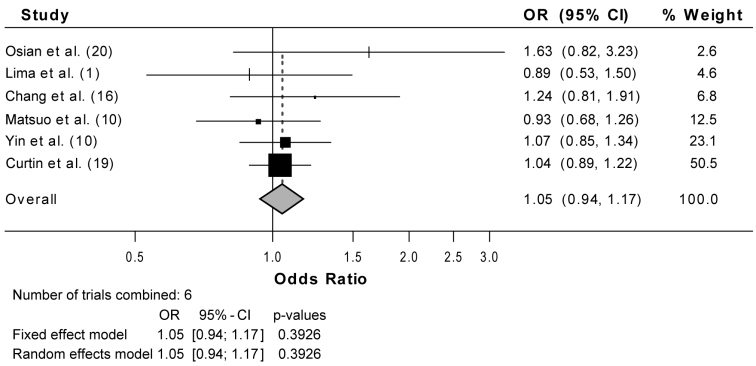

Figure 5b.
MTHFR 1298 AC vs AA (restricted criteria)

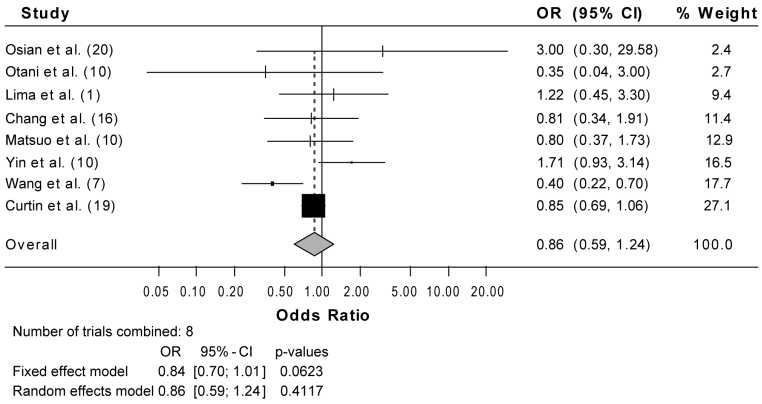

Figure 6a.
MTHFR 1298 CC vs AA (open criteria)

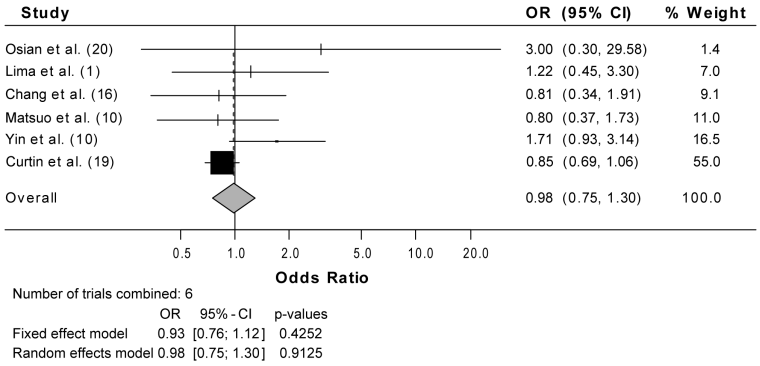

Figure 6b.
MTHFR 1298 CC vs AA (restricted criteria)

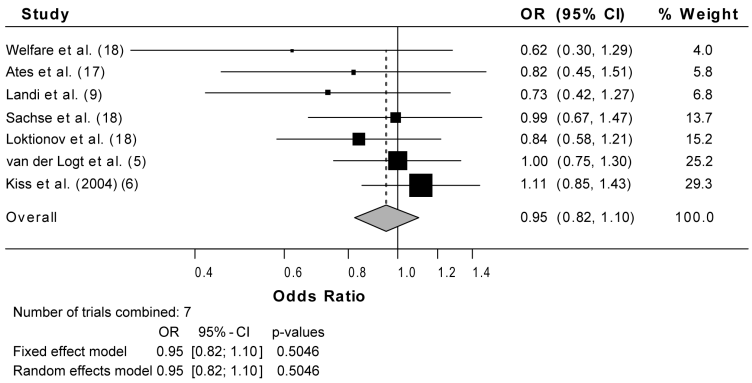

Figure 7a.
GSTP GG vs AA (open criteria)

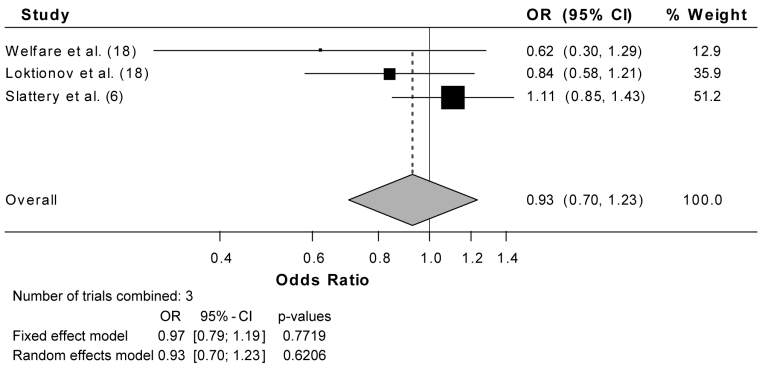

Figure 7b.
GSTP GG vs AA (restricted criteria)

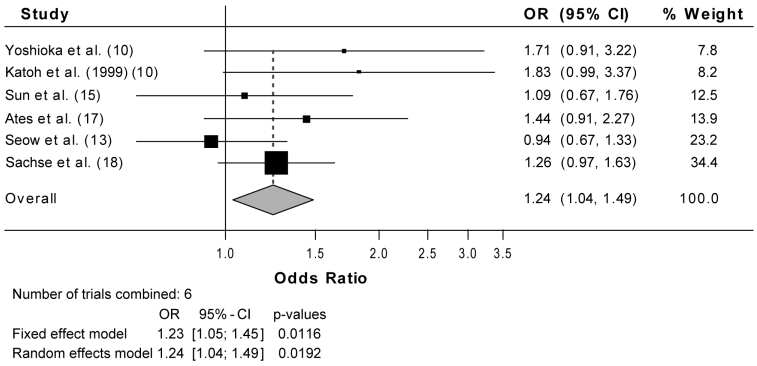

Figure 8.
GSTP AG vs AA (open criteria)

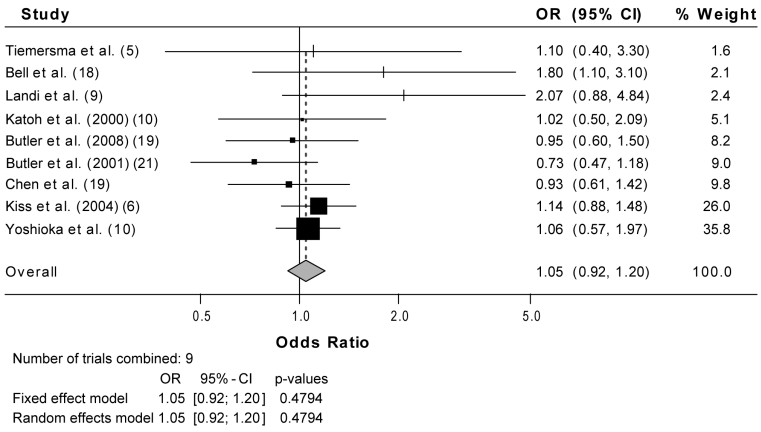

Figure 9.
NAT1 rapid acetylators (open criteria)

Table 1

PubMed* search strategy and results

Search terms:	Citations **
"Colorectal Neoplasms"[Mesh] AND "Polymorphism, Genetic" [Mesh]	1778
"Colorectal Neoplasms"[Mesh] AND "Polymorphism, Genetic" [Mesh] NOT "Adenomatous Polyposis Coli"[Mesh] NOT "Colorectal Neoplasms, Hereditary Nonpolyposis"[Mesh]	1424
"Colorectal Neoplasms"[MeSH] NOT "Adenomatous Polyposis Coli"[Mesh] NOT "Colorectal Neoplasms, Hereditary Nonpolyposis"[Mesh] AND "family history"	841
"Colonic Polyps"[Mesh] AND "Polymorphism, Genetic"[Mesh]	37
"Colonic Polyps"[Mesh] AND "family history"	104

* http://www.ncbi.nlm.nih.gov

Limits: English language, human studies

** Numbers are not mutually exclusive for articles retrieved.

Table 2

Number of studies meeting quality criteria, by single nucleotide polymorphism.*

	GSTM1	*GSTT1*	*GSTP1*	*MTHFR* C677T	*MTHFR* A1298C	*NAT1*
Total number of articles	20	15	11	10	8	9
Genotyping methods described	20 (100%)	15 (100%)	11 (100%)	10 (100%)	8 (100%)	9 (100%)
Studies include only histologically confirmed colon cancer cases	12 (60%)	9 (60%)	8 (73%)	9 (90%)	7 (88%)	4 (44%)
Persons with known colorectal cancer mutations excluded	7 (35%)	5 (33%)	4 (37%)	6 (60%)	6 (75%)	2 (22%)
Hardy Weinberg equilibrium assessed **	6 (30%)	5 (33%)	5 (45%)	8 (80%)	8 (100%)	0
Controls matched to cases on at least age and sex **	6 (30%)	3 (20%)	3 (27%)	7 (70%)	6 (75%)	3 (33%)
Blinding for assays **	2 (10%)	2 (13%)	1 (9%)	9 (90%)	5 (63%)	4 (44%)
Total meeting stricter criteria for inclusion	5 (25%)	3 (20%)	3 (27%)	6 (60%)	6 (75%)	1 (11%)

* Criteria are not mutually exclusive.

** Because of the small number of studies meeting these criteria, the criteria were not included for determining the restricted set of studies. The information is presented here for informational purposes.

Table 3

Association summaries of polymorphisms and publication bias

Polymorphism (PM)	Full meta-analysis, odds ratio, 95% CI For exposure (PM) & outcome variable (colorectal cancer)	Publication bias p-value for full metaanalysis*	Restricted meta-analysis, odds ratio, 95% CI For exposure (PM) & outcome variable (colorectal cancer)	Publication bias p-value for restricted meta-analysis*
NAT1	1.05 (0.92-1.20)	0.40	N/A**	N/A**
MTHFR 677CT	1.00 (0.90-1.10)	0.65	1.00 (0.90-1.12)	0.09
MTHFR 677 TT	0.86 (0.69-1.07)	0.10	0.87 (0.68-1.11)	0.19
*MTHFR*1298 AC	1.00 (0.87-1.15)	0.46	1.04 (0.94-1.17)	0.35
*MTHFR*1298 CC	0.86 (0.59-1.24)	0.62	0.98 (0.75-1.30)	0.19
GSTT1	1.32 (1.08-1.61)	0.46	1.30 (1.04-1.63)	0.60
GSTM1	1.21 (1.09-1.33)	0.65	1.16 (0.94-1.43)	1.00
GSTP1 AG	1.24 (1.03-1.49)	0.19	N/A***	N/A***
GSTP1 GG	0.95 (0.82-1.10)	0.02	0.93 (0.70-1.23)	0.12

*Null hypothesis: no publication bias.

**Only one study included; test not performed.

***No studies were included; test not performed.