Original article

Lack of consistency in the associations of *Helicobacter pylori* seropositivity with *Se* and *Le* polymorphisms among Japanese

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Abstract

Background. In our previous study, a marked association between *Helicobacter pylori* seropositivity and functional polymorphisms of the *secretor* and *Lewis* genes (odds ratio [OR], 0.32; 95% confidence interval [CI], 0.14–0.70 for *se/se* genotype relative to *Se/Se* genotype; and OR, 10.3; 95% CI, 3.16–33.8 for high-risk group defined by the combination of *Se* and *Le* relative to low-risk group) had been observed for 239 non-cancer Japanese outpatients of the gastroenterology clinic (OGC) undergoing gastroscopy at Aichi Cancer Center Hospital.

Methods. The present study was a confirmatory study to examine the association for 679 first-visit outpatients (FVO) of all clinics at the same cancer hospital and for 465 health checkup examinees (HCE) in the same city.

Results. The associations between *H. pylori* seropositivity and the *Se* and *Le* genotypes were nonsignificant or even in the opposite direction among the FVO (OR, 1.52; 95% CI, 1.00–2.32 for *se/se* genotype relative to *Se/Se* genotype; and OR, 0.77; 95% CI, 0.43–1.40 for the high-risk group defined similarly to the previous study), and among the HCE (OR, 1.25; 95% CI, 0.75–2.07; and OR, 1.07; 95% CI, 0.50–2.26, respectively). The discrepancy between the previous and present results was not explained by the difference in the distributions of age, sex, smoking, and *H. pylori* seroprevalence.

Conclusion. Even in the same ethnic group, different sources of subjects may demonstrate inconsistent findings due to an unidentified effect modification. Inconsistent findings have rarely been reported by the same research group, but they are very important to understand the whole picture of the association under study.

Key word *Helicobacter pylori* · Polymorphisms · *secretor* gene · *Lewis* gene · PCR-CTPP

Introduction

It is evident that *Helicobacter pylori* infection is related to several digestive diseases, including stomach cancer [1–3]. Acquisition of infection seems to occur primarily in childhood through the oral-oral or fecal-oral route, mainly from the family [4–6]. There is no doubt that environmental factors, especially sanitary conditions, determine the infection rate in the population. However, some individuals do not develop persistent infection even under the same poor sanitary condition, suggesting that host factors play a role in the infection and its maintenance. In addition, a twin study demonstrated that monozygous twins had a higher concordant rate of *H. pylori* infection than dizygous twins [7], indicating the importance of genetic traits.

Until recently, the information on genetic factors of the host and *H. pylori* infection was very limited; only HLA genotype and ABO blood type had been examined in relation to *H. pylori* infection [8–10]. In the past year, significant associations between *H. pylori* seropositivity and DNA sequence variants of the host were reported for the following genes; *secretor* (*Se* or *FUT2*) and *Lewis* (*Le* or *FUT3*) [11], *interleukin 1B* (*IL-1B*) [12,13], *myeloperoxidase* (*MPO*) [14], and *tumor necrosis factor- α* (*TNF- α*) [15]. The *Se* and *Le* genes encode the enzymes related to the synthesis of H type I (type O substance of ABO blood type) and Lewis b (*Le*^b) antigens, respectively. These gene products are the ligands for blood-group antigen-binding adhesin (BabA) of *H. pylori*. The other three genes encode proinflammatory cytokines and enzymes, which could be attributed to a condition of the persistent *H. pylori* infection.

Concerning *Se* and *Le* polymorphisms, the biological background for the significant association seems clear. BabA on the surface of the *H. pylori* membrane combines H type I and *Le*^b expressed on the gastric epithe-

lial cells [16–18]. The attachment of *H. pylori* to the epithelium causes a series of pathogenic processes in the gastric mucosa [19]. H type I is synthesized from the precursor by the *Se* enzyme, and then converted to *Le^b* by the *Le* enzyme. The *Le* enzyme also metabolizes the precursor of H type I to Lewis a (*Le^a*) antigen [20]. Low activity of *Se* enzyme and high activity of *Le* enzyme is considered to prevent the synthesis of H type I and *Le^b*, which may result in a reduced chance of *H. pylori* infection. It has been demonstrated that the *sej* and *se5* alleles of the *Se* gene and the *le1* and *le2* alleles of the *Le* gene have negligible or no functional activity [21–23]. In a previous study [11], we reported that low seropositivity of anti-*H. pylori* IgG antibody was associated with low expression alleles of *Se* and high expression alleles of *Le*.

Consistency, defined as the repeated observation of an association in different populations under different circumstances, is one of the criteria for establishing a causal association. It is not uncommon that epidemiologic studies of genetic polymorphisms show inconsistent results, possibly due to effect modification by genetic background and/or lifestyle. Therefore, we conducted confirmatory studies to examine whether the previously observed association of *Se* and *Le* genotypes with *H. pylori* seropositivity [11] could be replicated in different study populations.

Subjects and methods

Study subjects

The subjects were sampled from first-visit outpatients of Aichi Cancer Center Hospital who participated in the Hospital-based Epidemiologic Research Program at Aichi Cancer Center — II (HERPACC-II) [24] in 2001, and from health checkup examinees who received a health checkup provided by Nagoya Municipal Government in 2000.

At the Aichi Cancer Center Hospital, approximately 20% of first-visit outpatients had a cancer, and about 60% of the first-visit outpatients donated 7 ml of peripheral blood after they had provided written informed consent for genetic polymorphism research [24]. The population enrolled in the study consisted of 679 first-visit outpatients who agreed to the blood donation (FVO group).

The other subject group consisted of residents of a western part of Nagoya City. Written informed consent for anonymous use of the residual blood for research purposes was obtained after blood was drawn for a health checkup. Typically, about 2 ml of blood was left after the routine tests for health checkup. Of 489 examinees invited to participate in the study, 468 (95.7%)

agreed to provide their residual blood for genotyping and other laboratory assays. Residual blood samples from 3 subjects were not available for DNA extraction [25]. The remaining 465 health checkup examinees (HCE group) were included in this study.

Laboratory tests

Peripheral blood of the FVO subjects at Aichi Cancer Center Hospital was separated into buffy coat and plasma on the day of blood sampling. The residual blood of the HCE subjects was similarly separated, 3 days after blood sampling. The buffy coat and plasma were stored at -40°C after the separation. DNA was extracted from the buffy coat with a QIAamp DNA Blood Mini Kit (QIAGEN, Valencia, CA, USA). The plasma was used for an anti-*H. pylori* IgG antibody test (“HM-CAP”, Enteric Products, Westbury, NY, USA). An enzyme-linked immunosorbent assay (ELISA) value of 2.3 or over was regarded as *H. pylori* infection-positive [26]. The antibody test was conducted by SRL (Tokyo, Japan).

The *Se* gene has six alleles; *Se1* (357C, 385A, 571C, and 628C), *Se2* (357T, 385A, 571C, and 628C), *sej* (357T, 385T, 571C, and 628C), *se3* (357C, 385A, 571T, and 628C), *se4* (357C, 385A, 571C, and 628T), and *se5* (conversion with pseudogene). Because both *Se1* and *Se2* have full enzyme activity, and *se3* and *se4* are very rare (0% out of 800 alleles) for Japanese [20], the genotyping was conducted to distinguish A385T for *sej* and the pseudogene for *se5* from *Se* (*Se1* and *Se2*) by means of a related duplex polymerase chain reaction with confronting two-pair primers (PCR-CTPP) method [27,28]. The primers were as follows; *se5* F0, 5'-TTT CAC TGC CAC CAG CAC CTG; *Se385* F1, 5'-ATC AAA GGC ACT GGG ACC CAG; *Se385* R1, 5'-GGA CGT ACT CCC CCG GGA T; *sej* F2, 5'-TGG AGG AGG AAT ACC GCC ACT; *sej* R2, 5'-GTC CCC TCG GCG AAC ATG G. The underlined letters are the bases for the A385T polymorphism, and the primer *se5*F0 was set for the pseudogene. Genomic DNA (30–100 ng) was used for each 25- μl reaction mixture, 0.18 mM dNTPs, 12.5 pmol each of the five primers, 2.5 μl glycerol, 0.5 units of AmpliTaq Gold, 2.5 μl GeneAmp 10 $\times$ PCR buffer containing 15 mM MgCl_2 (Perkin-Elmer, Foster City, CA, USA). PCR conditions were 10 min of initial denaturation at 95°C , followed by 35 cycles of 1 min at 95°C , 1 min at 61°C , and 1 min at 72°C , and a 5-min final extension at 72°C . Amplified DNA was visualized on a 2% agarose gel containing ethidium bromide. The amplified band with 284 bp, 216 bp, and 353 bp represents the *Se* allele (385A), *sej* (385T), and *se5* allele, respectively, while a common band of 460 bp appeared for the *Se* and *sej* alleles, as demonstrated in Fig. 1a.

Fig. 1a,b. Representative examples of agarose gel electrophoresis. **a** *Se* A385T and conversion type (*se5*) polymorphisms. Lane M, 100-bp ladder marker; lane 1, A/A (*Se/Se*); lane 2, T/T (*se/se*); lane 3, *se5se5* (*se/se*); lane 4, A/T (*Se/se*); lane 5, T/*se5* (*se/se*); and lane 6, A/*se5* (*Se/se*). **b** *Le* T59G polymorphism. Lane M, 100-bp ladder marker; lane 1, T/T (*Le/Le*); lane 2, T/G (*Le/le*); and lane 3, G/G (*le/le*).

The *Le* gene has four alleles; *Le* (59T, 508G, and 1067T), *le1* (59G, 508A, and 1067T), *le2* (59G, 508G, and 1067A), and *le3* (59G, 508G, and 1067T). Because *le3* is very rare (0.5% out of 800 alleles) for Japanese [20], T59G was genotyped to distinguish *le1* and *le2* from *Le* by PCR-CTPP. The primers were: F1, 5'-CCA TGG ATC CCC TGG GTG; R1, 5'-CCA CCA GCA GCT GAA ATA GCC; F2, 5'-CGC TGT CTG GCC GCA CT; and R2, 5'-GAA GGT GGG AGG CGT GAC TTA. Genomic DNA (30–100ng) was used for each 25- μ l reaction with 0.18mM dNTPs, 25pmol each of the primers, 0.5 units of AmpliTaq Gold, and 2.5 μ l GeneAmp 10 $\times$ PCR buffer containing 15mM MgCl₂ (Perkin-Elmer). The PCR conditions were same as those for the *Se* genotyping, except for annealing at 66°C. Amplified were a 329-bp band for the T allele (*Le*) and an 81-bp band for the G allele (*le1* and *le2*), while a common band of 373bp appeared for both alleles (Fig. 1b).

The present study had been approved by the Ethics Committee at Aichi Cancer Center before the study started (Ethics Committee Approval Numbers 41-2 and 11-12).

Statistical analysis

An unconditional logistic model was applied for estimating odds ratios (ORs), using a computer program (STATA version 7; STATA, College Station, TX, USA). ORs were adjusted for age as a dichotomous variable (<50 years vs $\geq$ 50 years). Goodness-of-fit of genotype distribution to Hardy-Weinberg equilibrium was examined by the "genhwi" procedure of STATA.

Results

Table 1 shows anti-*H. pylori* IgG seropositivity according to sex and age group for FVO and HCE subjects. The ages ranged from 18 to 79 years for the FVO and from 32 to 85 years for the HCE subjects. The seropositivity increased with age, and leveled off around age 50 years for both groups of subjects. The age-specific rate for men was slightly higher or equal to that for women, except for the 18-to-29-year-olds in the FVO group and the 30-to-39-year-olds in the HCE group.

The percentages of self-reported current smokers at study entry were 35.9% in men and 13.2% in women in the FVO group, and 38.6% in men and 5.6% in women in the HCE group. The seroprevalence of anti-*H. pylori* antibody in the male FVO group was 59.3% for current smokers ($n = 113$), 61.0% for former smokers ($n = 136$), and 62.1 for never smokers ($n = 66$); and in the female FVO group, the seroprevalences were 45.8% ($n = 48$), 53.3% ($n = 30$), and 46.7% ($n = 285$), respectively. The corresponding prevalences in the HCE group were 59.2% ($n = 49$), 85.7% ($n = 7$), and 69.9% ($n = 71$) for men; and 42.1% ($n = 19$), 20.0% ($n = 5$), and 51.6% ($n = 314$) for women. The difference in *H. pylori* seroprevalence between current smokers and never smokers was not statistically significant for either sex according to subject group by a χ^2 test.

The *Se* genotype was determined for all subjects, except for 1 HCE subject, and the *Le* genotype was determined for 663 (97.6%) of the 679 FVO subjects and 424 (91.2%) of the 465 HCE subjects. The genotype frequencies of each polymorphism are shown in Table 2. The genotype distribution was in Hardy-Weinberg equilibrium ($\chi^2 = 0.77$; $P = 0.38$ for *Se*, and $\chi^2 = 2.19$; $P = 0.14$ for *Le* among the outpatients; and $\chi^2 = 1.45$;

Table 1. Seroprevalence of anti-*Helicobacter pylori* antibody by study population, age, and sex

Age (years)	First-visit outpatients (FVO)				Health checkup examinees (HCE)			
	Male		Female		Male		Female	
	<i>n</i>	<i>H. pylori</i> + % ^a	<i>n</i>	<i>H. pylori</i> + %	<i>n</i>	<i>H. pylori</i> + %	<i>n</i>	<i>H. pylori</i> + %
18–29	14	7.1	25	8.0	0	—	0	—
30–39	24	33.3	69	27.5	9	11.1	40	17.5
40–49	35	54.3	66	47.0	7	42.9	51	39.2
50–59	97	66.0	113	52.2	10	60.0	97	58.8
60–69	91	69.2	60	68.3	65	75.4	116	56.9
70–79	54	66.7	31	61.3	32	71.9	30	63.3
80–85	0	—	0	—	4	50.0	4	50.0
Total	315	60.6	364	47.0	127	66.1	338	50.6

^a *H. pylori* + %, Percentage of anti-*H. pylori* antibody-positive subjects**Table 2.** Genotype distributions of *Secretor* and *Lewis* genes

Genotype	First-visit outpatients (FVO)		Health checkup examinees (HCE)	
	<i>n</i> (%)	<i>H. pylori</i> + ^a (<i>H. pylori</i> + %)	<i>n</i> (%)	<i>H. pylori</i> + (<i>H. pylori</i> + %)
<i>Secretor</i> gene				
<i>SeSe</i>	170 (25.0)	79 (46.5)	139 (30.0)	69 (49.6)
<i>Sese</i>	328 (48.3)	180 (54.9)	218 (47.0)	126 (57.8)
<i>sese</i>	181 (26.7)	103 (56.9)	107 (23.1)	59 (55.1)
Total	679 (100)	362 (53.3)	464 (100)	254 (54.7)
<i>Lewis</i> gene				
<i>LeLe</i>	353 (53.2)	189 (53.5)	235 (55.7)	126 (53.4)
<i>Lele</i>	251 (37.9)	132 (52.6)	155 (36.6)	87 (56.1)
<i>lele</i>	59 (8.9)	36 (61.0)	33 (7.8)	20 (60.6)
Total	663 (100)	357 (53.8)	424 (100)	233 (55.0)

^a *H. pylori*+, Anti-*H. pylori* antibody-positive

$P = 0.23$ for *Se*, and $\chi^2 = 1.12$; $P = 0.29$ for *Le* among the examinees). When both sexes were combined, a χ^2 test for 2 (seronegative vs seropositive) by 3 (genotypes) showed no statistically significant associations between the *H. pylori* seropositivity and the genotypes of *Se* and *Le* for either group, although the seroprevalence was higher among individuals with *sese* or *lele* than among those with *SeSe* or *LeLe*, respectively.

Table 3 shows the sex-and-age-adjusted ORs for *H. pylori* seropositivity by study population. The OR for FVO subjects with the *se* allele (*Sese* or *sese*) was significantly higher than that for those with *SeSe*, and the adjusted OR for HCE subjects with *Sese* was marginally significant (95% confidence interval, 1.00–2.46). The *Le* genotype showed no significant association with seropositivity for either study population.

We also estimated ORs for *H. pylori* seropositivity according to risk groups created based on the results of our previous study [11]; low-risk (*sese* & *LeLe*), moderate risk (combinations other than low- or high-risk groups), and high-risk (*SeSe* & *lele*, *SeSe* & *Lele*, and

Sese & *lele*) groups. In the present study, no statistically significant association was observed between the combined genotype risk group and *H. pylori* seropositivity.

Discussion

This study did not reproduced the association of *H. pylori* seropositivity with the *Se* and *Le* polymorphisms observed in the preceding study of 239 outpatients (118 males and 121 females) who underwent gastroscopy at the Gastroenterology Clinic (OGC) of Aichi Cancer Center Hospital [11]. The subjects of the previous study included 95 (39.7% of 239) participants who stated that they were taking medication for diseases (not confirmed by their medical records); 23 subjects were taking medication for gastric/duodenal ulcer, another 23 for so-called gastritis, and 59 for miscellaneous diseases or conditions (i.e., some subjects were taking medication for more than one disease; no. of diseases = 105). The *H. pylori* seropositivity was 72.1% for *SeSe*, 64.6% for

Table 3. Odds ratios (ORs) and 95% confidence intervals (95% CIs) for *H. pylori* seropositivity

Genotype	First-visit outpatients (FVO)		Health checkup examinees (HCE)	
	cOR ^a (95% CI)	aOR ^b (95% CI)	cOR ^a (95% CI)	aOR ^b (95% CI)
<i>Secretor</i> gene				
<i>SeSe</i>	1 (Reference)	1 (Reference)	1 (Reference)	1 (Reference)
<i>Sese</i>	1.40 (0.97–2.03)	1.51 (1.02–2.22)	1.39 (0.91–2.13)	1.57 (1.00–2.46)
<i>sese</i>	1.52 (1.00–2.32)	1.50 (0.97–2.33)	1.25 (0.75–2.07)	1.29 (0.76–2.19)
<i>Sese/sese</i>	1.44 (1.02–2.04)	1.50 (1.05–2.16)	1.34 (0.90–2.00)	1.47 (0.97–2.23)
<i>Lewis</i> gene				
<i>LeLe</i>	1 (Reference)	1 (Reference)	1 (Reference)	1 (Reference)
<i>Lele</i>	0.96 (0.70–1.33)	0.98 (0.70–1.37)	1.12 (0.74–1.68)	1.06 (0.69–1.63)
<i>lele</i>	1.36 (0.77–2.39)	1.31 (0.73–2.37)	1.34 (0.64–2.83)	1.40 (0.63–3.07)
<i>Lele/lele</i>	1.03 (0.76–1.39)	1.03 (0.75–1.42)	1.15 (0.78–1.70)	1.11 (0.74–1.67)
Combined risk group ^c				
Low	1 (Reference)	1 (Reference)	1 (Reference)	1 (Reference)
Moderate	0.71 (0.45–1.11)	0.79 (0.49–1.28)	1.28 (0.71–2.34)	1.50 (0.81–2.81)
High	0.77 (0.43–1.40)	0.77 (0.43–1.40)	1.07 (0.50–2.26)	1.07 (0.49–2.34)

^aCrude OR^bSex-and-age-adjusted OR by dichotomous variables (male vs female and age <50 years vs ≥50 years)^cBased on the results of a previous study [11]. Low-risk, *sese* & *LeLe*; high-risk, *SeSe* & *lele*, *SeSe* & *Lele*, and *Sese* & *lele*; moderate risk, the other combinations

Sese, and 45.1% for *sese*, and 55.6% for *LeLe*, 68.4% for *LeLe*, and 76.5% for *lele*. When the genotypes were combined, the sex-and-age-adjusted OR relative to individuals with *sese* & *LeLe* (low-risk group) was 10.21 (95% confidence interval, 2.98–34.96) for individuals with *SeSe* & *lele*, *SeSe* & *LeLe*, or *Sese* & *lele* (high-risk group) and 3.34 (1.36–8.20) for the remaining individuals (moderate risk group). Even when the 23 ulcer patients and 23 gastritis patients were excluded, a strong association was observed (the ORs became larger). The observed association was consistent with a biologically plausible hypothesis described in the “Introduction” in this article.

The lack of or the opposite associations observed for individuals in the same geographic area as those in the previous study [11] were a surprise to us. We expected that such associations with genetic polymorphisms would be consistent within one ethnic group, especially in the same geographic area. For example, we observed consistent associations between *H. pylori* seropositivity and the *IL-1B* C-31T polymorphism among Japanese in three datasets [12,13,29], between the *Dopamine Receptor D2* polymorphism and smoking habit in two datasets [30,31], and between the *IL-1B* C-31T polymorphism and smoking habit in two datasets [25].

In epidemiologic studies of genetic polymorphisms and disease risk, inconsistent results are not rare among ethnically and/or geographically diverse groups even after eliminating the possibilities of various biases. Confounding by race/ethnicity, also known as population stratification, is a well-recognized phenomenon observed in a study that includes subjects from different ethnic groups. Inconsistent findings could result from

other factors, such as random errors, unknown effect modification, dilution of the association strength, and their combinations. We discuss below these potential explanations for the inconsistency observed among three studies conducted in Aichi, Japan.

The first potential explanation is random error. Our previous study for OGC had 239 subjects, including 90 seronegative individuals. Seventeen of 61 subjects with *SeSe* and 4 of 17 subjects with *lele* were seronegative. The main purpose of the present study was to confirm the promising finding of the previous study [11], using 527 seronegative and 617 seropositive subjects (in total 1144) sampled from the FVO and HCE groups. Because the 95% confidence interval of the OR for the high-risk group of OGC did not overlap with those observed in the present study, a random error may be not the cause of the inconsistent results. The present study with larger sample size should provide statistically more stable results than our previous study of OGC, if there were neither effect modifiers nor different degrees of dilution.

The second possibility is unknown effect modification, or interaction between risk factors (genetic or environmental). Gene-gene interactions and gene-environment interactions have been documented for several diseases [32–35]. We observed that smoking habit was not associated with *H. pylori* seropositivity in FVO [29] and HCE subjects [13], while it was associated with the seropositivity in OGC subjects [12]. Although there is a controversy concerning the association between *H. pylori* infection and smoking habit [36], the inconsistent results on smoking seem to be due to an effect modification; the association tends to be stronger

for patients with gastrointestinal complaints than for healthy residents; stronger in high-prevalence than in low-prevalence areas; and stronger in populations with a high smoking prevalence than in populations with a low smoking prevalence [37]. In this study, the *sese* genotype percentages (26.7% in the 679 FVO subjects and 23.1% in the 464 HCE subjects) were similar to or slightly higher than those observed for Japanese in other areas (24.8% of 141 subjects [22], 21.3% of 231 subjects [11], and 18.5 of 400 subjects [20]), and the *lele* genotype percentages (8.9% of 663 FVO subjects and 7.8% of 424 HCE subjects) were also similar to those in other studies (10.0% of 400 subjects [20] and 7.1% of 239 subjects [11]). The association between *H. pylori* seropositivity and *Se* and *Le* polymorphisms, however, may be modified by unknown factors.

Dilution of the association occurs when subjects whose outcome under study is not associated with risk factors of interest (e.g., those whose seropositivity is not influenced by *Se* and *Le* genotypes) are mixed with subjects for whom the outcome and risk factors are associated. An overall OR for the whole study population would become closer to unity (i.e., null association) with an increased proportion of subjects in the former group.

In the present study, there was no information on the mixture of individuals whose *H. pylori* seropositivity was not associated with the *Se* and *Le* genotypes. However, it is possible that *H. pylori* with BabA might be more prevalent in OGC than in FVO and HCE subjects. Because H type I and Le^b antigens play a role in the attachment of BabA-positive *H. pylori* to gastric epithelial cells [18,19], the *Se* and *Le* genotypes do not influence the susceptibility for those with BabA-negative *H. pylori*. If BabA-positive *H. pylori* was associated with the hospital visit of *H. pylori* infected individuals due to severe gastritis [38], the marked association observed only for OGC would be explained. In Japan, the *BabA2* gene encoding BabA was reportedly highly prevalent (84.9% of 152 strains) in patients who underwent gastroscopy [39], but no data are available for healthy residents. We could not examine for this hypothetical possibility, because *H. pylori* samples from our subjects were not available.

In summary, the association between *H. pylori* seropositivity and functional polymorphisms of *Se* and *Le* was not reproduced in the present study of 679 first-visit outpatients and 465 health checkup examinees. Because the association is biologically plausible, the inconsistent findings observed for the same ethnic group in the same geographic area may be due to unknown effect modification and/or dilution of association strength, although they could be due to other types of bias. Like other inconsistent findings observed for polymorphisms and disease risk, potential effect modifiers remain to be

identified. Inconsistent findings are often unreported, but we believe it is important to report them to reduce the chance of reaching wrong conclusions due to publication bias.

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