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Original article:

Genetic polymorphism of Clara cell secretory protein 16KDa (CC16) and susceptibility to asthma, a meta-analysis

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ABSTRACT

Clara cell secretory protein 16KDa (CC16) is the major secretory protein of the Clara cells. The gene encoding CC16 has been implicated as potential susceptibility gene for asthma. Published studies between genetic polymorphism of CC16 at position 38 and asthma risk are controversial. Most of these studies are based on small sample size. To clarify the role of the A38G CC16 polymorphism on the risk of developing asthma, we carried out a meta-analysis of the research published between January 1988 and August 2005. In the present study, 11 case-control studies with 2362 subjects (1262 controls and 1100 patients) were eligible for meta-analysis. The fixed-effects method was used when there was no significant heterogeneity between the results of the individual studies being pooled, whereas, the random-effects method was used when there was heterogeneity between the studies. The overall ORs of the asthma risk were not associated with the CC16 genotypes (For AG vs GG: OR=1.09, 95% CI: 0.90-1.32; For AA vs GG: OR=1.05, 95% CI: 0.79-1.39; For AG+AA vs GG: OR=1.09, 95% CI: 0.91-1.30). It should be noted that similar results were obtained when we used the stratified data, adjusted for age and smoking status of the subjects. In conclusion, the CC16 A38G polymorphism was not associated with asthma risk in the present meta-analysis.

Keywords: Asthma, CC16, susceptibility, meta-analysis, polymorphism

INTRODUCTION

A genetic component to asthma and atopy has been suggested (Malerba and Pignatti, 2005). Genome-wide searches and candidate gene studies have identified many genes and regions that potentially influence asthma susceptibility (Daniels et al., 1996; Malerba and Pignatti, 2005). The human chromosome 11q12-13 is one of the regions which reported to show linkage to atopy and asthma (Daniels et al., 1996; Malerba and Pignatti 2005). The gene encoding Clara cell secretory protein 16 KDa (CC16; synonyms: Uteroglobin and CC10) has been mapped to this region (Hay et al., 1995). The protein sequence is evolutionary conserved among mammals, suggesting an important, if not critical, role in mammalian physiology (Hashimoto et al. 1996). It is the most common protein in the bronchoalveolar lavage fluid (BALF) of healthy non-smokers (Bernard et al., 1992a). CC16 levels are significantly lower in asthmatic patients (Shijubo et al., 1999; Van Vyve et al., 1995) and in smoker subjects

(Bernard et al., 1992b; Robin et al., 2002). There is growing evidence from in vitro and in vivo studies suggesting that this protein plays an important protective role in the ling as an anti-inflammatory agent (Dierynck et al., 1995; Lesur et al., 1995; Milele et al., 1987). Mice deficient in CC16 expression were found to exhibit a higher susceptibility to oxidant-induced lung injury and an excessive inflammatory response (Johnston et al., 1997; Mango et al., 1998).

Taken together, these evidences, suggest that CC16 plays a protective role in the lung, and it is possible that low activity variants of CC16 may be associated with inflammatory lung disorders such as asthma.

The gene encoding CC16 is 5 kb in length, and is comprised of a 550-bp 5'-untranslated region (5'UTR) and three exons (Hay et al. 1995). An adenine/guanine polymorphism at position 38 (A38G) downstream from the transcription start site was previously reported (Laing et al., 1998a). It is reported that the 38A allele has lower transcription levels than the 38G allele, suggesting a potential loss of anti-inflammatory activity in the lung for individuals with the 38A allele (Laing et al., 2000). Several studies have examined the association between the A38G polymorphism of CC16 and susceptibility to asthma. The results of these studies, however, are not consistent (Bakhu et al., 1998; Baldini et al., 1998; Candelaria et al., 2005; Gao et al., 1998; Gui et al., 2003; Kalyoncu et al., 2003; Laing et al., 1998a; Laing et al., 1998b; Mansur et al., 2002; Mao et al., 1998; Saadat et al., 2004; Sanak et al., 1999; Sengler et al., 2003; Sharma and Ghosh 2004).

One of the main aims of genetic epidemiology is to understand the genetic contribution to complex diseases such as asthma. One of the most popular study designs in this area is a molecular association study in which a polymorphism is linked to the disease outcome in cases and controls. These studies are often limited by small sample sizes, so there is a role for meta-analysis in pooling these studies, particularly to detect the small effect sizes that may be associated with these polymorphisms. Most of the studies investigating the association between CC16 polymorphism and risk of asthma are based on small sample size (Candelaria et al., 2005; Kalyoncu et al., 2003; Laing et al., 1998a; Mansur et al., 2002; Saadat et al., 2004). In order to clarify the effect of CC16 genotype on the risk of developing asthma, we carried out a meta-analysis using published data from 1996 up to the August 2005, to obtain more precise estimates of risk.

MATRIALS AND METHODS

Identification of studies:
Studies published between January 1998 and August 2005 with information of CC16 genetic polymorphism and the risk of asthma were identified using electronic database, MEDLINE (National Library of Medicine, Washington, DC, USA). Search terms were "asthma" and "CC16" or "Clara cell protein". Additional articles were also checked using the references cited in these publications.

Articles selected for analysis were studies with case-control design and their primary references, which had no obvious overlap of cases with other studies. Five studies were not including in the analysis because their cases showed overlap with other reports, presented as an abstracts in seminars or published in Chinese (Bakhu et al., 1998; Baldini et al., 1998; Gui et al., 2003; Laing et al., 1998b; Sanak et al., 1999;). Study of Mao et al. (1998) also excluded

from the analysis because the authors investigated the association between asthma and an intragenic variant of CC16. Studies of Gao et al. (1998) and also Sengler et al. (2003) that included different patients groups were considered as four studies in the analysis. The application of these criteria yielded 11 case-control studies eligible for meta-analysis (Candelaria et al., 2005; Gao et al., 1998; Kalyoncu et al., 2003; Laing et al., 1998a; Mansur et al., 2002; Saadat et al., 2004; Sengler et al., 2003; Sharma and Ghosh 2004).

In all of the studies, the polymorphism was determined by PCR assays. The PCR was performed according to the method introduced by Laing et al (1998a). Amplification products were digested with *Sau96I*. Restriction digestion of amplified DNA samples showed three altered digested patterns identifying heterozygous and homozygous subjects for both 38A and 38G alleles. Genetic polymorphism was defined as AA (absence of restriction site on both alleles), GG (presence of restriction site on both alleles) or AG (heterozygous). It is suggested that the 38G allele is more likely to be the wild type allele (Laing et al., 1998a).

Statistical analysis:
For control group of each study allelic frequency was calculated. For control group of each study, the observed frequencies of the CC16 genotypes were assessed for Hardy-Weinberg equilibrium using the χ^2 statistic.

The odds ratio (OR) of asthma associated with the CC16 genetic polymorphism was re-calculated for each study, and their corresponding 95% confidence intervals (CI) were estimated. The results might not be exactly the same as those of some studies as different criteria were used in the statistical analysis. A low-risk genotype (GG) was used as the baseline for calculation ORs. For comparing the allelic distribution between patients and controls, the 38G allele was used as the baseline for calculation ORs.

To take into account the possibility of heterogeneity across the studies, a statistical test for heterogeneity was performed based on the Q statistic, in which a P-value greater than 0.05 suggested a lack of heterogeneity (DerSimonian and Laird 1986). We carried out meta-analysis using both a fixed-effects and a random-effects model. The fixed-effects method assumes no significant heterogeneity between the results of the individual studies being pooled, whereas, the random-effects method allows for such heterogeneity. The fixed-effects and random-effects methods were used by Mantel-Haenszel (Mantel and Haenszel 1959) and DerSimonian and Laird methods (DerSimonian and Laird 1986), respectively. In Tables we report the results of fixed-effects method and if there is heterogeneity between studies, we report the results of random-effect method. The analyses were also conducted on the subgroups of studies based on age and smoking status of the subjects. The p-value less than 0.05 considered statistically significant.

RESULTS

Comparing CC16 allelic frequencies between patients and controls:
We identified 11 eligible studies, including 2362 subjects (1100 patients, and 1262 healthy controls) in relation to the A38G polymorphism of CC16 and risk of asthma, which are summarized in Table 1. From these, 5, 5 and 1 studies were carried out in Asian and European countries and in Australia, respectively (Table 1). The numbers in the case-control studies varied considerably (range 96 to 510 individuals). The frequency of the 38A allele varied in the

control participants, from 18.2 percent (in Iran) to 52.4 percent (in India).

Table 1: Distributions of 38A and 38G alleles of CC16 in controls and asthmatic patients

Study	Country	Age	Controls				Cases			
			Smoker	G	A	Frequency of A (%)	G	A	OR	95% CI
Laing et al., 1998a	Australia	Child	None	68	24	26.1	76	58	2.16	1.17-4.02
Gao et al., 1998	England	Adult	Some	186	114	38.0	169	81	0.78	0.54-1.13
Gao et al., 1998	Japan	Adult	Some	122	78	39.0	121	79	1.02	0.67-1.56
Gao et al., 1998	Japan	Adult	Some	122	78	39.0	122	78	1.00	0.66-1.53
Mansur et al.,2002	England	Adult	Some	52	30	36.6	113	61	0.94	0.52-1.68
Sengler et al., 2003	Germany	Child	None	163	73	30.9	113	55	1.09	0.70-1.70
Sengler et al., 2003	Germany	Child	None	163	73	30.9	144	64	0.99	0.65-1.52
Kalyoncu et al., 2003	Turkey	Adult	? *	81	29	26.4	55	27	1.37	0.70-2.69
Saadat et al., 2004	Iran	Adult	None	139	31	18.2	121	49	1.82	1.06-3.13
Sharma & Ghosh 2004	India	Adult	None	239	263	52.4	256	262	0.93	0.72-1.20
Candelovia et al., 2005	Denmark	Adult	Some	547	285	34.3	51	45	1.69	1.08-2.65

* The smoking status of subjects was not mention in the article.
** The 38A and 38G are the low and high risk alleles, respectively.

Table 2 shows the association between frequency of the 38A allele and susceptibility to asthma. Test for heterogeneity between studies showed heterogeneity between the studies (Q statistic=19.648, df=10, P<0.05). Therefore, the random-effects model was used for calculation of OR and its 95% CI (Table 2). The overall OR of the asthma risk associated with the 38A allele of the CC16 gene was 1.09 (95% CI: 0.96-1.23) which is not statistically significant.

Table 2: Summary of meta-analysis of case-control studies of CC16 polymorphism and the risk of asthma, distribution of 38A allele between cases and controls

	Q statistic	df	OR	95% CI
All of studies	19.648*	10	1.09	0.96-1.23
Adulthood	13.806	6	1.05	0.91-1.21
Childhood	5.056	2	1.21	0.93-1.58
Non-smokers	10.97*	4	1.12	0.94-1.32
Smokers	7.72	4	1.03	0.85-1.24

***** There is heterogeneity between studies P<0.05.

Table 2 also summaries the results of the stratified meta-analysis. Subgroup analysis, regarding age and smoking status of the subjects were carried out. Studies were stratified according to the age of subjects (adulthood and childhood). Three studies investigated the association between the frequency of CC16 alleles and risk of asthma in children. The total sample size was 255 with asthma and 164 controls. Eight studies assessed the association between the frequencies of CC16 alleles and asthma risk in adults. The sample size was 845 for asthma and 1098 for controls. No heterogeneity was detected between the studies (For adulthood: Q statistic= 13.806, df=6, P>0.05; For childhood: Q statistic=5.056, df=2, P>0.05). Statistical analysis showed there is no significant association between the frequency of the 38A allele and asthma risk in the both groups (For adulthood OR=1.05, 95% CI: 0.91-1.21; For childhood OR=1.21, 95% CI: 0.93-1.58).

In order to investigate the possible interaction between cigarette smoking

and the genotypes of CC16, the studies were stratified according to the smoking status of the subjects. Study of Kalyoncu et al. (2003) did not mention the smoking status of their subjects; therefore, we excluded this study for the analysis. Five studies determined the association between the frequencies of CC16 alleles and asthma in non-smokers. Total sample size was 599 with asthma and 500 control subjects. There was significant heterogeneity between the studies (Q statistics=10.97, df=4, $P<0.05$). Therefore the OR was calculated using random-effects method. Five studies investigated the association between the frequency of CC16 alleles and asthma risk in smoker subjects. Total sample size was 460 subjects with asthma and 707 healthy persons as control group. There was no heterogeneity between the studies (Q statistics=7.72, df=4, $P>0.05$). Association between the frequency of CC16 alleles and asthma was not statistically significant (For smokers: OR=1.03, 95% CI: 0.85-1.24; For non-smokers: OR= 1.12, 95% CI: 0.94-1.32).

Comparing the CC16 genotypic frequencies between patients and controls:

Table 3 summaries CC16 genotypic frequencies in patients and controls of the studies included in the meta-analysis. Here the study of Laing et al. (1998a) was excluded, because the raw data of the genotypes frequencies was not available. The CC16 genotype frequencies in all of the control groups were in Hardy-Weinberg equilibrium ($P>0.05$, data not shown). Here we analyzed the following comparisons:

a) AG genotype versus GG genotype

b) AA genotype versus GG genotype

c) AA+AG genotypes versus GG genotype

In pooled and in all of the subgroup analyses, there was no identifiable evidence of heterogeneity in the analyses of CC16 and asthma risk (Table 4). The overall ORs of the asthma risk were not associated with the CC16 genotypes (For AG vs GG: OR=1.09, 95% CI: 0.90-1.32; For AA vs GG: OR=1.05, 95% CI: 0.79-1.39; For AG+AA vs GG: OR=1.09, 95% CI: 0.91-1.30). It should be noted that when we used the stratified data, based on age and smoking status of subjects, same results were obtained (Table 4).

Table 3: Distributions of CC16 genotypes in controls and asthmatic patients

Study	χ^2 for HW*	Controls			Cases			AG vs GG		AA vs GG		AA+AG vs GG	
		GG	GA	AA	GG	GA	AA	OR	95% CI	OR	95% CI	OR	95% CI
Gao et al., 1998	0.88	55	76	19	54	61	10	0.82	0.48-1.40	0.54	0.21-1.35	0.76	0.45-1.27
Gao et al., 1998	0.25	36	50	14	36	49	15	0.98	0.51-1.88	1.07	0.41-2.77	1.00	0.54-1.86
Gao et al., 1998	-	36	50	14	41	40	19	0.70	0.36-1.35	1.19	0.48-2.94	0.81	0.44-1.49
Mansur et al.,2002	0.11	17	18	6	37	39	11	1.00	0.41-2.39	0.84	0.23-3.09	0.96	0.42-2.17
Sengler et al., 2003	0.09	57	49	12	35	43	6	1.43	0.76-2.68	0.81	0.25-2.62	1.31	0.72-2.39
Sengler et al., 2003	-	57	49	12	48	48	8	1.16	0.64-2.10	0.79	0.27-2.30	1.09	0.62-1.91
Kalyoncu et al., 2003	0.02	30	21	4	18	19	4	1.51	0.59-3.87	1.67	0.30-9.36	1.53	0.63-3.76
Saadat et al., 2004	0.74	58	23	4	45	31	9	1.74	0.85-3.57	2.90	0.75-12.1	1.91	0.98-3.75
Sharma and Ghosh 2004	1.67	62	115	74	68	119	72	0.94	0.60-1.48	0.89	0.54-1.46	0.92	0.61-1.40
Candelovia et al., 2005	2.19	173	201	42	13	25	10	1.66	0.78-3.54	3.17	1.19-8.39	1.92	0.95-3.94

* Testing for Hardy-Weinberg equilibrium, df=1, P>0.05

** The GG is the low risk genotype.

Table 4: Summary of meta-analysis of case-control studies of CC16 polymorphism and the risk of asthma, distribution of the CC16 genotypes between cases and controls

		AG vs GG			AA vs GG			AA+AG vs GG		
Study	**df**	**Q statistic**	**OR**	**95% CI**	**Q statistic**	**OR**	**95% CI**	**Q statistic**	**OR**	**95% CI**
All of studies	9	8.51	1.09	0.90-1.32	12.49	1.05	0.79-1.39	10.96	1.09	0.91-1.30
Adulthood	7	7.41	1.04	0.83-1.29	11.83	1.09	0.81-1.49	10.47	1.06	0.87-1.30
Childhood	1	0.25	1.28	0.84-1.96	0.001	0.80	0.37-1.74	0.21	1.19	0.79-1.78
Non-smokers	3	2.75	1.19	0.91-1.58	3.40	0.97	0.66-1.44	3.96	1.16	0.89-1.51
Smokers	4	3.71	0.94	0.69-1.27	8.36	1.10	0.72-1.70	5.37	0.98	0.75-1.29

Note: There is no heterogeneity between studies P<0.05.

DISCUSSION

The overall goal of meta-analysis is to combine the results of previous studies to arrive at summary conclusions about a body of research. It is most useful in summarizing prior research when individual studies are small and they are individually too small to yield a valid conclusion. In the present study, 11 case-control studies were found eligible for meta-analysis (Candelaria et al., 2005; Gao et al., 1998; Kalyoncu et al., 2003; Laing et al., 1998a; Mansur et al., 2002; Saadat et al., 2004; Sengler et al., 2003; Sharma and Ghosh 2004). There was no evidence of heterogeneity between studies in the present meta-analysis (Tables 2 and 4). The present results show no association between the A38G polymorphism of the CC16 gene and risk of asthma, thus providing evidence against a major role played by this polymorphism in the predisposition to asthma. This finding is not consistent with the location of CC16 on human chromosome (Hay et al., 1995), genome-wide searches (Daniels et al., 1996; Malerba and Pignatti, 2005), physiological functions of CC16 (Bernard et al., 1992a; Dierynck et al., 1995; Johnston et al., 1997; Lesur et al., 1995; Mango et al., 1998; Milele et al., 1987; Shijubo et al., 1999; Van Vyve et al., 1995;), and alterations of CC16 levels in the serum of smoker subjects (Bernard et al., 1992b; Robin et al., 2002) and asthmatic patients (Shijubo et al., 1999; Van Vyve et al., 1995). This may be due to following reasons:

First: The human CC16 gene maps to an important genetic region which has been linked by various studies to asthma and its related phenotypes (Cookson et al., 1989; Daniels et al., 1996). In addition to the CC16 gene, several other known potential candidate gene(s) is the target remains a matter of debate. Polymorphisms in other genes located on human 11q (FcεRI-β and GSTP1) have been associated with total serum IgE, atopy and BHR (Fryer et al., 2000; Hill et al., 1995; Shirkawa et al., 1994). Additionally the region is likely to harbour other genes yet to be identified.

Second: There is few studies showed significant association between A38G polymorphism and asthma risk (Candelovia et al., 2005; Laing et al., 1998a; Saadat et al., 2004). However, several studies (Gao et al., 1998; Kalyoncu et al., 2003; Mansur et al., 2002; Sengler et al., 2003; Sharma and Ghosh 2004) and also the results of the present study failed to replicate the association between the A38G polymorphism of CC16 and asthma. The A38G polymorphism of CC16 and the intragenic microsatellite repeat polymorphism intron 1 at the haplotype level was investigated in a case-control study in India (Sharma and Ghosh 2004). The authors also attempted to evaluate the association between these markers individually and at the haplotype level to asthma status. They found the significant association between the above-mentioned markers and asthma risk (Sharma and Ghosh 2004). It is probable that this is not an effect of the single nucleotide polymorphism of CC16, but instead, reflects a common haplotype that includes this allele. At least in part, the phenomenon of linkage disequilibrium between the 38A allele and other loci involved in developing asthma might be into account.

Third: Asthma is a complex disorder caused by the additive effects of many genes and environmental factors and their interactions which each having only a small effect on developing the phenotype. It is reported that the GST genes and other genes involved in the oxidation stress have been described to be involved in asthma and atopy (Fryer et al., 2000; Tamer et al., 2004). Gene

polymorphisms of the several members of GST superfamily (such as GSTP1, GSTT1, GSTM1) have been associated with asthma and atopy (Fryer et al., 2000; Saadat et al., 2004; Tamer et al., 2004). We have recently reported a strong association between the combination genotypes of genetic polymorphisms of CC16, GSTM1, and GSTT1 and asthma in a case-control study (Saadat et al., 2004). We found that the concurrent lack of the GSTM1 and GSTT1 genes increased asthma risk about 10.23 ($P<0.001$). On the other hand we found a weak association between 38A allele and risk of asthma. However, when combination of CC16, GSTM1, and GSTT1 genotypes was analyzed, we found that individuals AA or AG genotypes when lack of both of the active genes of GSTM1 and GSTT1 posed more than a 22-fold odds of asthma. The additive effect of genetic polymorphisms of GSTM1 and GSTT1 was reported for developing several types of cancers, such as gastric and colorectal cancers (Saadat and Saadat 2001) and asthma (Invaschenko et al., 2002). It is probable that the CC16 polymorphism has very small effect on the susceptibility of asthma, but its combination with other genes, results on increased the association between genetic polymorphisms and asthma. Therefore, it would be interesting to investigate the additive effects of several genes (either located on chromosome 11q or located on the other chromosomes) involved in the developing asthma.

Forth: Suppose that we wished to test that presence of 38A allele has the effect of increasing the risk of asthma about 1.10 (OR). With 80% power, for detecting a difference at the 0.05 level, we would require about 15200 subjects (7600 patients and 7600 controls). The number of subjects, in the present meta-analysis is about 2600, which is much lower than the required samples. This warrants further investigation in larger studies.

It might be concluded that the relationship between the CC16 genotypic variation and susceptibility to asthma is still an open question. The above mentioned points suggest that in the future research in this field should take great care in the interaction between healthy risk factor (life-style conditions, such as smoking behavior, alcohol and air pollution) and combination genotypes of CC16 and other genes (such as GSTM1, GSTT1, GSTP1 genes). Also additive effect of involved genes should be investigated.

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