cancer varied by *GSTM1* status in a Chinese population with the mutant allele frequency of CYP1A1 Ile-Val polymorphism intermediate between Japanese and Caucasian population [11]. For non-Asian populations, the relevance of *CYP1A1 Msp* I and *GSTM1* polymorphisms to lung cancer is questionable [12], given their low prevalence in both lung cancer and general populations. However, combined *CYP1A1* and *GSTM1* genotype is thus a potential predictor of genetic susceptibility to smoking-related lung cancers in populations where *CYP1A1* alleles are common.

2.1.4. Microsomal epoxide hydrolase

Microsomal epoxide hydrolase (mEH) is an important phase II biotransformation enzyme and it is highly expressed in several human tissues including the lung, where it catalyzes the hydrolysis of various epoxides and reactive epoxide intermediates into less reactive and more water soluble dihydrodiols, which are then excreted from the body [13–15]. Hence, mEH is a protective enzyme involved in general oxidative defenses against a number of environmental substances [15–17]. However, mEH is also involved in the xenobiotic activation of tobacco carcinogens. Combined with CYP, mEH can metabolize PAHs into highly mutagenic and carcinogenic diol epoxides [18–20]. Thus, the activation or inactivation effects of mEH depend on the specific compounds being metabolized.

The *mEH* gene is located on chromosome 1q42.1. There are two polymorphisms that affect enzyme activity in the human *mEH* gene. One variant is characterized by substitution of histidine for tyrosine (Tyr113His) in exon 3 (*EH3*) and the other is substitution of arginine for histidine (His139Arg) in exon 4 (*EH4*), conferring low and high enzymatic activity, respectively [21].

Lower activity *mEH* genotypes were associated with decreased lung cancer risk in several studies (Table 1). French study found that lower activity of *mEH3* genotype (His/His) was a protective factor for lung cancer [22]. Three Caucasian studies [15,24,25], two Chinese studies [23,26] and a study of Wu et al. among African–Americans and Mexican–Americans [27] found, however, no significant relationship between *mEH3* genotype and lung cancer risk. A significant protective effect of low activity *mEH3* genotype was observed among African–Americans [25] and Spaniards [24]. The seven case-control studies of lung cancer and *mEH3* genotype included 2626 subjects (1010 lung cancer cases and 1616 controls). The overall OR was 0.96 (95% CI = 0.66–1.39).

For *mEH4*, six of seven studies could not found a significant association and the direction of the association was different among them [15,22,23,25–27]. However, the exon 4 polymorphism associated with a significantly increased risk of lung cancer among

Chinese and Mexican–Americans [23,27]. The overall OR among the seven case-control studies with nine different ethnic populations (1010 lung cancer cases and 1616 controls) was 1.44 (95% CI = 1.03–2.00), which was significantly different than 1.0 (Table 2). A test for heterogeneity suggested no significant heterogeneity.

When combine polymorphisms *EH3* and *EH4*, low activity genotype was significantly associated with a decreased risk of lung cancer among French Caucasian [22] in Table 2. However, other studies among Caucasian populations [15,24,25] and among African–Americans [25] did not confirm the association. Although a Taiwanese study also found that low mEH activity genotype was not associated with decreased risk of lung cancer (OR = 1.03, 95% CI = 0.66–1.61), low activity genotype was significantly associated with a decreased risk of squamous cell carcinoma (OR = 0.51, 95% CI = 0.27–0.96) [28]. The relationship between *mEH* genotypes and lung cancer risk has not been studied in a large number of subjects. Recently, no relationship between the low activity genotype and lung cancer risk was found by a large American study [29]. It also indicated that cumulative cigarette smoking exposures play pivotal roles in the association between both *mEH* polymorphisms and lung cancer risk, altering the direction of risk (in the case of combined low activity genotype in both *EH3* and *EH4*) from a risk factor (OR = 1.59, 95% CI = 0.80–3.14) in non-smokers to a protective factor (OR = 0.45, 95% CI = 0.22–0.93) in heavy smokers [29]. The six case-control studies in seven different ethnic populations of lung cancer and the combined genotype included 4381 subjects (1818 lung cancer cases and 1563 controls). The overall OR was 0.96 (95% CI = 0.68–1.34).

Differences in associations between ethnic subgroups or between study populations can result from linkage disequilibrium with additional allelic variants that modulate overall enzyme activity and may be present in different frequencies in the different groups or linkage disequilibrium with another gene that is causally related to lung cancer. Joint effects among *mEH* genotype, other genetic polymorphisms and cigarette smoking should be investigated in additional studies.

2.1.5. NAD(P)H quinone oxidoreductase (DT-diaphorase)

NAD(P)H quinone oxidoreductase 1 (NQO1), formerly referred to as DT-diaphorase, is a cytosolic enzyme catalyzing the two-electron reduction of quinone substrates. NQO1 either metabolically activates or detoxifies carcinogens present in cigarette smoke. BP is one of the most important carcinogens and the formation of BP quinone–DNA adduct is prevented by NQO1 [30]. In contrast, carcinogenic heterocyclic amines present in smoke are metabolically activated by NQO1 [31]. Therefore, this enzyme is thought to be

genetic susceptibility to lung cancer may in part be determined by inter-individual variations in the genetic factors associated with cigarette smoking. Advances in molecular biology have led growing interest in investigation of biological markers, which may increase/decrease predisposition to smoking-related carcinogenesis.

Genetic difference is known for substances in tobacco smoke absorption, their metabolism and for their interactions with receptors. In this paper, we discuss the relationship between genetic polymorphisms and lung cancer, with special emphasis on the genes of drug metabolizing enzymes other than well-investigated cytochrome P450 (CYP) and glutathione S-transferase (GST) isozymes, DNA repair genes and the p53 tumor suppressor gene.

2. Materials and methods

A search of the English literature using the National Library of Medicine MEDLINE and essential search terms for the years 1985–2001 was undertaken to identify all published articles or abstracts in which the frequency of each genotype was determined for human lung cancer by PCR method. Additional articles were identified through the references cited in the first series of articles. Articles selected for meta-analysis were case-control in design, published in the primary literature and had no obvious overlap of subjects with other studies.

To take into account the possibility of heterogeneity between studies, a random effects model was used the deviation of the overall odds ratios (ORs). This model assumes that the studies in questions are a random sample of a hypothetical population of studies taking into account within- and between-study variability. Overall ORs were calculated using Stata Statistical Software Release 7.0, 2001 (Statacorp, Texas, USA).

2.1. Metabolic polymorphisms

2.1.1. Cytochrome P450s

Cigarette smoke contains several thousand chemicals, of which about 50 compounds are known carcinogens, including polycyclic aromatic hydrocarbons (PAHs), aromatic amines and N-nitroso compounds. Some of these compounds are reactive carcinogens, but most are procarcinogens, which need to be activated by phase I enzymes such as those encoded by the CYP supergene family, and converted into reactive carcinogens. All these reactive carcinogens can bind to DNA and form DNA adducts capable of inducing mutations and initiating carcinogenesis. CYPs are a multigene superfamily of mixed function monooxygenases. Based on sequence homology, the CYP superfamily is divided into 10 subfamilies, CYP1–CYP10. Subfamilies CYP1,

CYP2, CYP3 and CYP4 are primarily involved in drug metabolism [5].

A positive association between development of lung cancer and the mutant homozygous genotype of CYP1A1 Msp I or CYP1A1 Ile-Val polymorphism has been reported in several Japanese populations but such an association has observed in neither Caucasians nor American–Africans. The CYP1A1 Msp I polymorphism has a higher variant allele frequency than the CYP1A1 Ile-Val polymorphism. The relationship between CYP2D6 gene and lung cancer remains conflicting and inconclusive. Several polymorphisms have been identified at the CYP2E1 locus. No definitive link between the polymorphisms of CYP2E1 and the risk of lung cancer has, however, been identified. Some studies on CYP2A6, CYP2C9 and CYP2C19 have indicated a relationship between lung cancer and the occurrence of a rare allele, although future research is needed in order to establish a significant relationship. Details were shown elsewhere [6].

The role of other CYP2 isoforms in lung carcinogenesis has not been sufficiently investigated yet.

2.1.2. Glutathione S-transferases

Following phase I reaction, phase II enzymes such as GSTs are responsible for detoxification of activated forms PAH epoxides. GSTs are constitutively found in a wide variety of tissues, with different characteristic patterns of GST isozymes. GST genes form a superfamily of at least 13 genes consisting of five distinct families, named alpha (GSTA), sigma (GSTS), mu (GSTM), pi (GSTP) and theta (GSTT). Certain genes within the GSTM, GSTT and GSTP subfamilies (GSTM1, GSTT1 and GSTP1) are polymorphic in humans and the levels of individual enzymes expressed can be influenced by induction and by genetic polymorphism. The role of GSTM, GSTT1 or GSTP1 polymorphism in modifying the lung cancer risk may be more limited than has been so far anticipated. Detailed were shown in elsewhere [6].

2.1.3. Combined phase I and II polymorphisms

Since genetic polymorphisms have been found for both phase I and II enzymes, risk assessment could be increased in sensitivity if polymorphisms in both phases of enzymes are taken into consideration as biomarkers for susceptibility to cancer. It is likely that an individual with the high risk genotype (either a genotype coding for a more active phase I enzyme or a less efficient phase II enzyme, or both of those) might be at higher risk of cancer than that with the opposite genotype (combination). The data in Asian population studies to date indicate the combined genotype between CYP1A1 Msp I and GSTM1 polymorphisms reveals higher OR for lung cancer than a single locus [7–10]. The association between the CYP1A1 Ile-Val polymorphism and lung

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Review

Genetic polymorphisms and lung cancer susceptibility: a review

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Abstract

Lung cancer is a major cause of cancer-related death in the developed countries and the overall survival rate has still an extremely poor. Cigarette smoking is an established risk factor for lung cancer although a possible role for genetic susceptibility in the development of lung cancer has been inferred from familial clustering of the disease and segregation analyzes. Everyone may have a unique combination of polymorphic traits that modify genetic susceptibility and response to drugs, chemicals and carcinogens. Developments in molecular biology have led to growing interest in investigation of biological markers, which may increase predisposition to lung carcinogenesis. Therefore, the high-risk genotype of an individual could be determined easily. As there are the great number of carcinogen-activating and -detoxifying enzymes, the variation in their expression and the complexity of exposures to tobacco carcinogens, the existence of multiple alleles at loci of those enzymes may result in differential susceptibilities of individuals. This review summarize data addressing the relationships of lung cancer to markers of genetic susceptibility genes, including metabolic polymorphisms other than well-investigated cytochrome P450s or glutathione S-transferases, DNA repair genes and the p53 tumor suppressor gene. Among genetic polymorphisms reviewed here, myeloperoxidase gene (a G to A mutation) and microsomal epoxide hydrolase exon 4 polymorphism (substitution of Arg for His) were significantly associated with lung cancer risk. As lung cancer is a multifactorial disease, an improved understanding of the interplay of environmental and genetic polymorphisms at multiple loci may help identify individuals who are at increased risk for lung cancer. Hopefully, in the future we will be able to screen for lung cancer susceptibility by using specific biomarkers. © 2002 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Lung cancer; Molecular epidemiology; Metabolic polymorphism; DNA repair gene; Meta-analysis; Germline polymorphism of tumor suppressor gene p53

1. Introduction

Lung cancer is one of the most common cancers worldwide and has the highest mortality rate among all cancers. There is irrefutable evidence that tobacco smoking causes bronchogenic carcinoma in approximately 85–90% of lung cancer victims. There is also evidence that environmental tobacco smoke exposure may cause lung cancer in life-long non-smokers [1,2].

The association between histological types and smoking was reviewed by and IARC working group [3] and concluded that squamous cell carcinoma, small cell carcinoma and adenocarcinoma were probably caused by smoking, although the relative risk was weaker with adenocarcinoma. The association of smoking and large cell carcinoma was not included in that IARC review and only recent studies provided data on this histological type of lung cancer.

Approximately one in 10 lifetime smokers develop lung cancer. This implies that host factors may influence individual susceptibility to tobacco smoke. A possible role for genetic susceptibility in the development of lung cancer has been inferred from familial clustering of the disease and segregation analyzes [4]. It is possible that

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Measurements of SCCA protein

SCCA protein was performed using a sensitive enzyme immunoassay. Briefly, cells were trypsinized and sonicated. After centrifugation, the supernatant was subjected to an automated assay kit (IMx, Dainabot, Tokyo, Japan). The IMx system measures the total amounts of SCCA protein including SCCA1 and SCCA2.

Immunocytochemical analysis

HBECs were acetone-fixed on slides. The sections were pretreated with 0.1% saponin (MERCK, Darmstadt, Germany) for 20 min at room temperature. The sections were probed with a monoclonal antibody against SCCA, mAb27 (Dainabot), followed by incubation with biotin-conjugated anti-mouse immunoglobulin antibody (Vector Laboratories), and then alkaline phosphatase-conjugated streptavidin (Dakopatts, Glostrup, Denmark). The signal was detected by the alkaline phosphatase method according to the manufacturer's instruction (Sigma). Specificity of the signal was confirmed by the observations that the signal was not elicited by treatment without the first antibody.

Measurement of serum level of SCCA in asthma patients

After obtaining informed consent, serum samples were collected from asthmatic and non-asthmatic children whose ages (1–13 year old) were matched. The asthmatic children had recurrent breathlessness and chest tightness, required on-going treatment and had physician-documented wheeze. One asthmatic child received inhaled corticosteroids. All of the asthmatic children except this subject received either oral adrenergic agents or theophyllines. Non-asthmatic children had no allergic history and no inflammatory symptoms. It was ascertained that all of the investigated children did not suffered from atopic dermatitis or malignancies. The study was approved by Saga Medical School's Ethics Committee. Measurement of SCCA was performed by ELISA as described above. Data were presented as median (inter-quartile range) and were analyzed using Mann–Whitney U-test for between group comparisons or Wilcoxon's Rank Sum Test for paired samples within a group.

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TABLE 2. Primers used for the quantitative PCR analysis of the identified genes

	Forward	Reverse	TaqMan® probe
SCCA1	5'-AGCCGCGGTCTCGTGC-3'	5'-GGCAGCTGCAGCTTCTG-3'	5'-AAGGCCTTTGTGGAGGTTACAGAGGAGGGA-3'
SCCA2	5'-AGCCACGGTCTCTCAG-3'	5'-GCAGCTGCAGCTTCCA-3'	5'-AAGGCCTTTGTGGAGGTCACTGAGGAGGGA-3'
DD96	5'-GCCTTTGCAGTCAACCACTTCTG-3'	5'-CTGTTCCCACCAGGACTCCAT-3'	5'-ATGATCCTGACCGTCGGAAACAAGGC-3'
KAL1	5'-AAGAGCCACAAGCCTATTGGC-3'	5'-AGCAGAAAGGTTCTCAGGCTTAGC-3'	5'-CCTGGGCGAAGCAGGTCATGTTCTTTCT-3'
Periostin	5'-AGCAAACCACCTTCACGGATC-3'	5'-GGTGCCAGCAAAGTGTATTCTCC-3'	5'-AATTAGGCTTGGCATCTGCTCTGAGGCC-3'
Tenascin C	5'-TTTCTGACATAACTCCCGAGAGC-3'	5'-AGATATGGGCAGTTCGTTCAGC-3'	5'-CGATGGGATCTTCGAGACCTTTACCATTG-3'
Carboxypeptidase M	5'-ACAGAGACGTTTGTCCTCTCTGC-3'	5'-ATGCCCCAGTTGCTTGAACAC-3'	5'-CCCTCGTGGCCAGTTACCCATTTGATA-3'
IL-13Rα2	5'-TGCTCAGATGACGGAATTTGG-3'	5'-TGGTAGCCAGAAACGTAGCAAAG-3'	5'-TGAGTGGAGTGATAAACAATGCTGGGAAGG-3'
Cathepsin C	5'-TCTCAGACCCCAATCCTAAGCC-3'	5'-CTGCAATAAGGTATGGGAAGCC-3'	5'-TCTTGTAGCCAGTATGCTCAAGGCTGTGAA-3'
Dioxin-inducible cytochrome P450	5'-TTATGAAGCCATGCGCTTCT-3'	5'-AAGACAGAGGTGTTGGCAGTG-3'	5'-CAGCTTGTGTGCCTGTCACTATTCCTCATG-3'
Carbonic anhydrase II	5'-CCTCTTCTGGAATGTGTGACCTG-3'	5'-CGGAATTTCAACACCTGCTCG-3'	5'-TCAAGGAACCCATCAGCGTCAGCA-3'
Endothelin-A receptor	5'-ACCTCTGCGCTCTTAGTGTTGAC-3'	5'-GCAGTTACCAAAGGAATCCCAA-3'	5'-TACAGAGCAGTTGCCTCCTGGAGTCGTGTT-3'

types at multiple loci in the IL-4RA gene could be more informative than the separate study of isolate SNPs. In particular, the above authors have shown that association with atopy is much stronger for two locus haplotypes than for separate SNPs. In the present paper, the authors report a study on the relationship between three IL-4RA SNPs and total IgE levels in the English population. The results of their two locus haplotype analysis confirm the higher informative value of haplotype analysis as compared to separate studies on single nucleotide polymorphisms.

Subjects and methods

One hundred and fifty subjects affected by atopic asthma and 150 healthy control subjects from the English population (Oxford district) were considered. These samples have already been analysed by the present authors' group with regard to the effect of several other polymorphisms on total IgE levels (10) and/or predisposition to atopic asthma (11). Total IgE levels, specific IgE levels for aeroallergens and the results of skin prick tests were available both for patients and controls. The usual limit of $100\,U\,mL^{-1}$ (approximately corresponding to the fiftieth percentile in this population) for total IgE levels was chosen for the analysis. Statistical analysis considering IgE as continuous variable cannot be performed with haplotypes since there is no direct correspondence between haplotype and individual (i.e. each individual carries two haplotypes). For this reason, the present authors considered a discrete subdivision of IgE distribution and compared the haplotype frequencies between IgE categories. A chi-square test of independence was performed by the SPSS computer program (12).

The determination of the Ile50Val, Ser478Pro and Gln551Arg polymorphism of the IL-4 receptor alpha chain was performed by restriction fragment length polymorphisms-polymerase chain reaction (RFLP-PCR). The two locus haplotypes frequencies are maximum likelihood estimates (MENDEL program, Department of Biostatistics, University of Michigan, Ann Harbor, MI, USA). Linkage disequilibrium (D') between SNP loci was measured according to Lewontin (13).

According to Ober et al. (9), probability values of chi-square tests of independence should not be adjusted for multiple comparison because the IL-4RA locus is considered an atopy susceptibility gene in the Caucasian population, and because the IL-4RA alleles and haplotypes are not independent of each other. Moreover, the present study could be considered as a partial replication of the study of Ober et al. in a separate outbred sample.

Nevertheless, in Table 3, the present authors show probability values which were both uncorrected and corrected for multiple comparisons.

Results

Table 1 shows the linkage disequilibrium between alleles at the three SNP loci in the whole sample. The present authors found no disequilibrium between the polymorphism in position 50 and the two other intracellular polymorphisms, while alleles at the two intracellular polymorphism in position 478 and 551 were in strong disequilibrium.

Table 2 shows the frequency of IL-4RA alleles at the three different SNPs in subjects with total IgE levels > $100\,U\,mL^{-1}$ and in subjects with IgE levels below this cut-off point. No significant association was present between total IgE levels and any of the three SNPs considered.

Table 3 shows the frequency of two locus haplotypes in relation to total IgE levels in the present sample. The distribution of haplotypes 50–478 showed a highly significant association with IgE level: most of the chi-square test data was derived from haplotype Val50/Pro478, which is much less frequent in subjects with IgE levels > $100\,U\,mL^{-1}$ than in those with IgE levels < $100\,U\,mL^{-1}$. Furthermore, the distribution of haplotypes 50–551 shows an association with IgE, but this is much less strong in comparison to haplotypes 50–478. As shown in Table 4, a lower frequency of the Val50/Pro478 haplotype is also present among the asthmatic subjects compared to the healthy controls, but the association is less strong in comparison to that observed for IgE levels. A similar trend in haplotype distribution is present in subjects positive for the prick tests for house dust mites in comparison to those who are negative, but the differences do not reach the level of statistical significance (data not shown).

Discussion

In line with the finding of Ober et al. (9), no linkage disequilibrium was found in the present authors' population between the extracellular polymorphism in position 50 and any of the two intracellular polymorphism considered. Furthermore, the higher informative value of haplotype analysis as compared to separate studies of single SNPs agrees with the finding of Ober et al. (9).

The three SNPs considered in the present paper have been all found to be associated with atopy in previous studies. In the study of Ober et al. (9), the association of the three separate SNPs with atopy or asthma was not statistically significant in all four populations considered. In the present study,

no association with the SNPs separately considered has been detected in the English population, but the association has been found with the two locus haplotypes between the extracellular polymorphism and the single intracellular polymorphisms.

The data reported by Ober et al. (9) show a tendency (which is not statistically significant in general) for the wildtype Ile50 to be under-transmitted, and for both the Ser478 and Glu551 wildtypes to be over-transmitted to affected individuals. On the basis of these indications, one would expected the haplotype Val50/Ser478 to be over-transmitted to affected individuals and Ile50/Pro478 to be under-transmitted. The present data are concordant with this expectation only for the Val50/Ser478 haplotype for both IgE and asthma analysis. Differences between populations have also been observed by Ober et al. (9) and this may be because of differences in the biochemical mechanism of the disease or in the distribution of alleles at modifier

loci influencing the predisposition to the disease. Moreover, the present authors have considered IgE levels while Ober et al. (9) considered the prick test as a marker of atopy.

The higher informative power of two locus haplotypes compared to SNPs could depend on the presence of unknown alleles associated with the disease and in linkage disequilibrium with the known ones. As Ober et al. (9) suggested, it seems worth searching for variations in the untranslated region (UTR) or intronic regions of the IL-4RA gene. In the present authors' case, the association of haplotypes composed of the extracellular polymorphism and the single intracellular ones could depend on the presence of other polymorphisms located between the 50 and 478 alleles: besides possible unknown variations in the intronic regions, the 375 and the 406 polymorphisms seem to be good candidates among the possible exonic ones.

Alternatively, the currently discovered variants

Table 1   Analysis of disequilibrium in the English population. Expected frequencies have been calculated by assuming random assortment among alleles: (χ^2) chi-square test for goodness of fit; (d.f.) degree of freedom; and (D') product of coupling haplotype frequency minus product of repulsion haplotype frequency

Haplotype	Frequency		n	χ^2	d.f.	P-value	D'
	Observed (± SE)	Expected					
IL4R-478 × IL4R-50							
Ile50/Ser478	0.471 ± 0.021	0.445					
Ile50/Pro478	0.072 ± 0.012	0.097					
Val50/Ser478	0.349 ± 0.020	0.375					
Val50/Pro478	0.107 ± 0.014	0.082	600	5.20312	3	0.15751	+0.0253
IL4R-551 × IL4R-50							
Ile50/Gln551	0.441 ± 0.021	0.426					
Ile50/Arg551	0.102 ± 0.014	0.116					
Val50/Gln551	0.344 ± 0.021	0.358					
Val50/Arg551	0.112 ± 0.015	0.098	600	1.4733	3	0.68845	+0.0143
IL4R-478 × IL4R-551							
Ser478/Glu551	0.766 ± 0.017	0.644					
Ser478/Arg551	0.054 ± 0.009	0.175					
Pro478/Glu551	0.019 ± 0.006	0.141					
Pro478/Arg551	0.160 ± 0.015	0.038	600	147.194	3	0.000006	+0.1246

Table 2. Allelic frequencies for the three IL-4RA polymorphisms in subjects with total IgE levels higher and lower than 100 U mL^{-1}: (χ^2) chi-square test; and (d.f.) degree of freedom

Haplotype	Total IgE			d.f.	P-value
	< 100 U mL^{-1}	> 100 U mL^{-1}	χ^2		
IL-4RA Ile50Val					
Ile50	0.547	0.540			
Val50	0.453	0.460	0.008	1	0.928
IL-4RA Ser478Pro					
Ser478	0.804	0.838			
Pro478	0.196	0.162	0.936	1	0.333
IL-4RA Gln551Arg					
Gln551	0.773	0.799			
Arg551	0.227	0.201	0.452	1	0.515
Number of subjects	161	139			

may have functional importance in influencing interactions between IL-4RA and variants in other molecules involved in IL-4 signalling. The function of molecules involved in signal transduction processes is highly dependent on their correct conformation, which could be influenced by variations at the 50 and 478 loci in turn. A similar possibility has been proposed by Kruse et al. (6) for the 478

and the 551 polymorphisms with regard to their effects on total IgE production. In the present study and that of Ober et al. (9), there is apparently no data in support for an interaction between the 478 and 551 loci with regard to their effect on markers of atopy.

Finally, it is possible that the interaction of a couple of SNPs from the same molecule may deter-

Table 3. Frequencies of two-locus haplotypes in subjects with total IgE levels higher and lower than 100 U mL^{-1} ($\pm$ SE): (χ^2) chi-square test of independence (haplotypes versus IgE level); (d.f.) degree of freedom; and (NS) not significant

Haplotype	Total IgE			d.f.	P-value	P*
	< 100 U mL^{-1}	> 100 U mL^{-1}	χ^2			
Haplotypes 50–478						
Ile50/Ser478†	0.491 ± 0.029	0.451 ± 0.031				
Ile50/Pro478†	0.055 ± 0.015	0.088 ± 0.019	11 238	3	0.01	0.05
Val50/Ser478‡	0.313 ± 0.027	0.387 ± 0.030				
Val50/Pro478‡	0.140 ± 0.021	0.073 ± 0.018	7075	1	0.01	0.025
Total number	322	278				
Haplotypes 50–551						
Ile50/Gln551†	0.454 ± 0.029	0.414 ± 0.031				
Ile50/Arg551†	0.133 ± 0.021	0.126 ± 0.022				
Val50/Gln551†	0.292 ± 0.027	0.384 ± 0.031				
Val50/Arg551†	0.121 ± 0.021	0.076 ± 0.019	7557	3	0.05	NS
Total number	322	278				
Haplotypes 478–551						
Ser478/Gln551†	0.757 ± 0.024	0.775 ± 0.025				
Ser478/Arg551†	0.047 ± 0.012	0.063 ± 0.015				
Pro478/Gln551†	0.016 ± 0.007	0.023 ± 0.009				
Pro478/Arg551†	0.179 ± 0.021	0.138 ± 0.021	2692	3	NS	NS
Total number	322	278				

*Probability value corrected for multiple comparisons.
†All haplotypes.
‡Val50/Pro478 versus others.

Table 4. Frequencies of two-locus haplotypes in subjects with and without asthma ($\pm$ SE): (χ^2) chi-square test of independence (haplotypes versus asthma); and (d.f.) degree of freedom

Haplotype	Subject			d.f.	P-value
	Non-asthmatic	Asthmatic	χ^2		
Haplotypes 50–478					
Ile50/Ser478*	0.493 ± 0030	0.449 ± 0030			
Ile50/Pro478*	0.067 ± 0016	0.077 ± 0018	7258	3	0.06
Val50/Ser478†	0.307 ± 0028	0.390 ± 0029			
Val50/Pro478†	0.133 ± 0021	0.083 ± 0018	3.88	1	0.05
Total number	300	300			
Haplotypes 50–551					
Ile50/Gln551*	0.450 ± 0030	0.431 ± 0030			
Ile50/Arg551*	0.109 ± 0020	0.095 ± 0020			
Val50/Gln551*	0.306 ± 0028	0.382 ± 0029			
Val50/Arg551*	0.133 ± 0022	0.091 ± 0019	5472	3	0.14
Total number	300	300			
Haplotypes 478–551					
Ser478/Gln551*	0.746 ± 0025	0.785 ± 0024			
Ser478/Arg551*	0.054 ± 0013	0.055 ± 0013			
Pro478/Gln551*	0.010 ± 0006	0.028 ± 0010			
Pro478/Arg551*	0.189 ± 0023	0.131 ± 0020	6669	3	0.08
Total number	300	300			

*All haplotypes.
†Val50/Pro478 versus others.

mine the final static or dynamic conformation of the molecule, which could be influenced by other polymorphisms, i.e. by the 'complex haplotype'. This interaction could also be influenced by the variability of other signal transduction molecules which could show differences among different populations.

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Human HTm4 is a hematopoietic cell cycle regulator

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Proper control of cell cycle progression is critical for the constant self-renewal, differentiation, and homeostasis of the hematopoietic system. Cells of all types share the common cell cycle regulators. The different expression patterns of common regulators, in a broad sense, define cell-type or lineage specificity. However, there remains the possibility of hematopoietic cell cycle regulators tailored to the demands of the hematopoietic system. Here we describe a novel protein, HTm4, which serves as a hematopoietic cell cycle regulator. Our data indicate that HTm4 is expressed in hematopoietic tissues and is tightly regulated during the differentiation of hematopoietic stem cells. It binds to cyclin-dependent kinase–associated (CDK-associated) phosphatase-CDK2 (KAP-CDK2) complexes, and the three proteins demonstrate similar patterns of cellular expression in human lymphoid tissues. HTm4 stimulates the phosphatase activity of KAP, and its C-terminal region is required for binding to KAP-CDK2 complexes and the modulation of KAP activity. Overexpression of HTm4 can cause cell cycle arrest at the G_0/G_1 phase. Thus, HTm4 is a novel hematopoietic modulator for the G_1-S cell cycle transition.

Introduction

Cell cycle progression and hematopoiesis are tightly coordinated (1, 2). The maintenance of proper hematopoiesis requires a balance between the proproliferative and antiproliferative properties of hematopoietic stem cells (HSCs). This balance can be biologically translated into the known capabilities of HSCs to self-renew, differentiate, and generate the entire population of hematopoietic cells of all lineages. During cell cycle progression, several antiproliferative signals are activated (3–6). Antiproliferative properties of HSCs serve important biological functions that, in addition to the preservation of integrity of genetic materials, include the preservation of multipotential, early progenitor cells (7, 8), their differentiation to the committed late progenitor cells (9, 10), and subsequent maturation to terminally differentiated hematopoietic cells.

The temporal expression of various cyclins and cyclin-dependent kinases (CDKs) is the foundation upon which many cell cycle modulators exert their influence (3–6). The inhibition of the kinase activities of CDK2, 4, and 6 is known to cause G_0/G_1 cell cycle arrest (reviewed in ref. 11), which is believed to be important for the differentiation and maturation of hematopoietic cells. This inhibition is achieved primarily through the binding of inhibitors to CDKs. The cyclin D-CDK4/6 complexes play an important role in the progression or restriction of cell cycle progression at the G_1 phase. Inhibition of cyclin D-CDK4/6 kinase activity by $p21^{Cip1/Waf1/Sdi1}$ and $p27^{Kip1}$ inhibitors at this restriction point causes G_0/G_1 cell cycle arrest. Cyclin E-CDK2 and cyclin A-CDK2 are responsible for the G_1-S phase transition (12) and S phase progression of the cell cycle, respectively. Both complexes are susceptible to negative regulation by $p21^{Cip1/Waf1/Sdi1}$ and $p27^{Kip1}$. Interference with cyclin E-CDK2 activity can cause G_0/G_1 cell cycle arrest. With the involvement of cyclin D-CDK4/6, the interplay between $p27^{Kip1}$ and cyclin E-CDK2 is complex, varying according to the dif-

ferent physiological conditions of the cell (reviewed in ref. 3). In a rapidly cycling cell, most of p27[Kip1] is degraded, and the residual amount of p27[Kip1] is sequestered by cyclin D-CDK4/6, thus alleviating its negative regulation upon cyclin E-CDK2. However, in the absence of mitogen, the concentration of p27[Kip1] protein increases while that of cyclin D decreases. This combined effect can result in rapid inactivation of cyclin E-CDK2 kinase and bring about G_0/G_1 cell cycle arrest in just one cell cycle. Due to their ability to modulate the kinase activities of CDKs, both p21[Cip1/Waf1/Sdi1] and p27[Kip1] have been implicated to be involved in many aspects of hematopoiesis (7-10), from stem cell kinetics to terminal differentiation and maturation of hematopoietic cells.

The monomeric, phosphorylated CDK2 has only a basal level of kinase activity. Activation of CDK2 kinase activity requires both the phosphorylation of threonine residue (Thr[160]) by CDK-activating kinase (CAK) and its binding to the cyclins (13-15). CAK can phosphorylate both CDK2 monomer and cyclins-CDK2 complex. Dephosphorylation of the active phosphorylthreonine residue of CDK2 inactivates its intrinsic kinase activity, representing yet another control mechanism for cell cycle progression. KAP, a CDK-associated phosphatase, can dephosphorylate Thr[160] in human CDK2 (16). It binds to CDK2 both in the presence and absence of cyclins (16-18), but can only dephosphorylate CDK2 in the absence of cyclins. The binding of KAP to the cyclins-CDK2 complex does not interfere with the kinase activity. It seems the function of KAP is to counter CAK in two ways: (a) dephosphorylation of monomeric CDK2 after it is phosphorylated by CAK and (b) interference with CAK kinase activity by binding to CDK2 or the cyclins-CDK2 complex. The biological significance of KAP is not as well established as that of CDK inhibitors. Nevertheless, it has been shown that exogenous expression of KAP slows the G_1 phase cell cycle progression in HeLa cells (17) and that aberrant KAP transcripts are detected in some hepatocellular carcinomas (19). These observations suggest that KAP has the same biological effects as that of CDK inhibitors, although their modes of action are different.

Although the expression patterns of some common cell cycle regulators vary among hematopoietic cells of different lineages (10, 20), all the components of cell cycle regulation thus far discussed are common to all cell types. Here we report a novel protein, HTm4, which acts as a hematopoietic cell cycle regulator. HTm4 is a member of HTm4/CD20/FcɛRIβ subfamily with four transmembrane domains (21). It is expressed in hematopoietic cells, including bone marrow cells. It has been found to be associated with atopic asthma (22). HTm4, KAP, and CDK2 form a physiologic complex, the in vivo presence of which is confirmed by their similar patterns of cellular expression in human lymphoid tissue. Our data demonstrate that HTm4 modulates the level of phosphorylation of CDK2 through its direct binding to KAP. The exogenous expression of HTm4 in U937 cells causes G_0/G_1 cell cycle arrest.

Thus, the cell cycle regulatory pathway involving the dephosphorylation of CDK2 has been expanded to include HTm4, and the participation of KAP in hematopoiesis is implicated.

Methods

In vitro cell culture and transfection. The isolation of bone marrow cells and the subsequent manipulations of the isolated stem cells were carried out as described previously (23). Cells at various stages of differentiation were isolated as single cells and then subjected to RT-PCR analysis for the expression of HTm4. The induction of differentiation of CD34+ stem cells was also done in the presence of 300 U/ml of granulocyte colony stimulating factor (G-CSF) or 3 U/ml of erythropoietin (Epo) for the period of time as specified (24). Transient expression of hemagglutinin-KAP (HA-KAP) was done as follows: 5 μg per 10[7] cells of pCMV-HA-KAP (18) and the control pCMV vectors were transfected into U937 and KU812, using Superfect reagent (QIAGEN, Valencia, California, USA). Twenty-four hours after transfection, cell lysates were prepared from both samples. U937 and KU812 were routinely maintained in RPMI-1640 medium supplemented with 10% (vol/vol) FBS, 2 mM glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin in an atmosphere of 5% CO_2, at 37°C.

Cell fractionation and preparation of cell lysates. Fractionation of the subcellular components of U937 or KU812 was done as follows: cells were resuspended in ice-cold PBS with Protease Cocktail Tablets (Roche Molecular Biochemicals, Mannheim, Germany) and homogenized in a Douce glass homogenizer. The extract was centrifuged at 200 g for 10 minutes at 4°C to pellet the nuclei. The supernatant was clarified further by centrifugation at 15,000 g for 30 minutes at 4°C. The pellet was considered to be the membrane fraction. Total lysate, nuclear, and membrane fractions were used for the Western blot analysis. For the production of U937 and KU812 cell lysates, we extracted cells (10[6] cells/ml) in PBS containing 0.5% Triton X-100 and Protease Cocktail Tablets at 4°C for 30 minutes. The clear lysates were collected after centrifugation at 4°C, 10,000 g for 30 minutes and kept on ice before use.

Expression analysis in single cells using RT-PCR. Individual cells were obtained by means of a Becton Dickinson Immunocytometry Systems (San Jose, California, USA) FACS cell sorter, by limiting dilution analysis as described by Taswell (25). cDNAs representative of the total mRNA isolated from individual cells were synthesized by a micro-reverse transcription reaction using oligo-dT primers as described by Berardi et al. (23). The PCR products were analyzed by Southern blot technique (26) using α[32]P-dCTP-labeled HTm4 cDNA probe.

Yeast two-hybrid system. Bait consisting of the last 25 amino acids of HTm4 (C_{HTm4}), from amino acid 190 to 214, was cloned into the vector pVJLII (27). The yeast strain L40 containing two reporter genes, HIS3 and LacZ, was used as the host for a two-hybrid assay. Plas-

mid vector was cotransformed with the human bone marrow cDNA-pGADGH library (CLONTECH, San Diego, California, USA) into L40. Transformants were plated onto synthetic medium lacking histidine, leucine, and tryptophan. After 10 days of growth, His⁺ colonies were patched onto selection plates and tested for β-galactosidase activity. Plasmid DNAs were isolated from positive clones and introduced by electroporation into *Escherichia coli* MH4 plated on leucine-free medium for the selection of the pGADGH construct. The putative positive cDNAs in pGADGH plasmid were then tested in a two-hybrid assay with irrelevant, negative controls. The negative controls used were MEK (28), byr (29), and lamin (27). The identified cDNA clones in pGADGH constructs were considered true positive when they were tested negative with the negative controls. To make sure that the interactions between HTm4 and its target proteins were not permutation dependent, the entire two-hybrid assay was repeated with constructs that had their expression vectors exchanged between two groups. In this case, HTm4 was subcloned into pGADGH while KAP into the vector pVJLII.

Generation of anti-HTm4 polyclonal Ab. A peptide, GSLQYPYHFQKHF, from the HTm4 sequence between the first and the second predicted transmembrane domains (amino acids 70 to 82), was synthesized with the amino-terminal cysteine added to facilitate coupling and purification procedures. The antigen was prepared and injected into 3- to 9-month-old rabbits to raise antiserum following established protocols (Zymed Laboratories Inc., South San Francisco, California, USA). The specific Ab's were purified using an epoxy-activated Sepharose 6B coupled with the peptide as described by the manufacturer (Amersham Pharmacia Biotech Europe GmbH, Uppsala, Sweden). Please note that our HTm4 Ab is only suitable for Western blot analysis and cell staining. Thus, for the immunoprecipitation of both the full-length and truncated HTm4 that are exogenously expressed in Flag-tagged forms, we used anti-Flag from Sigma Chemical Co. (St. Louis, Missouri, USA).

Flow cytometry analysis. To determine the cellular expression (cell surface versus intracellular localization) of HTm4, intact U937 (10^7 cells/ml) was labeled with 2 μg/ml of polyclonal anti-HTm4 for 20 minutes, followed by the incubation with goat anti-rabbit IgG-FITC-conjugated Ab. For the staining of intracellular proteins, cells were permeabilized with the Fix & Cell Permeabilization Kit, as described by the manufacturer (Caltag Laboratories Inc., Burlingame, California, USA). The flow cytometry analysis was performed on a FACScan flow cytometer (Becton Dickinson Immunocytometry Systems). For the absorption with the peptide (amino acids 70–82) described above, 2 μg/ml of HTm4 Ab was treated with 10 μg of peptide at room temperature with continuous rotation for 30 minutes before the staining of cells.

Immunohistochemistry and immunoprecipitation. The cell preparation for immunohistochemistry study was per-

formed as described (30) with some modifications. Briefly, U937 cells were allowed to adhere to polylysine precoated coverslips for 30 minutes, fixed with 2% formaldehyde in PBS, and permeabilized in 0.1% saponin and 1× PBS. All subsequent washes and Ab hybridization were carried out in the presence of saponin-PBS buffer. Cells were prehybridized with 2% BSA, incubated with 0.2 μg/ml anti-KAP followed by 1 μg/ml anti-HTm4 for 20 minutes at room temperature. The positive staining was visualized with goat anti-mouse IgG Texas Red-conjugated or goat anti-rabbit IgG FITC-conjugated secondary Ab (Jackson ImmunoResearch Laboratories Inc., West Grove, Pennsylvania, USA). For immunoprecipitation, cell lysates equivalent to 5×10^5 cells in 0.5 ml of lysis buffer, 5 μg of Ab, and 40 μl of protein G Sepharose beads (Sigma Chemical Co.) were used per reaction.

Western blot analysis. Enhanced chemiluminescence detection reagents were purchased from Amersham Pharmacia Biotech (Piscataway, New Jersey, USA). Primary mAb's used were anti-KAP, anti-CDK2, both from BD Transduction Laboratories (San Diego, California, USA), anti-HA from Covance Research Products Inc. (Richmond, California, USA), and anti-Flag from Sigma Chemical Co. Secondary Ab's, horseradish peroxidase-conjugated (HRP-conjugated) goat anti-rabbit and rabbit anti-mouse Ab's, were from Amersham Pharmacia Biotech (Piscataway, New Jersey, USA). All primary Ab's were used at 1 μg/ml and the secondary Ab's at 1:5,000 dilution.

Isolation of KAP using GST-C$_{HTm4}$ fusion protein. pGEX-4T-C$_{HTm4}$ vector was used for the production of GST-C$_{HTm4}$. This expression vector contained the C-terminal region of HTm4 that coded for the amino acid 190 to 214 (C$_{HTm4}$), with in-frame reading for GST protein at the 5′ end, using pGEX-4T-1 vector (Amersham Pharmacia Biotech, Piscataway, New Jersey, USA). The production and purification of GST-C$_{HTm4}$ was done according to the protocols specified by the manufacturer (Amersham Pharmacia Biotech, Piscataway, New Jersey, USA). GST protein without the fusion partner was used as control. One milliliter each of U937 and KU812 lysates was incubated with 5 μg of GST-C$_{HTm4}$ or GST coupled to glutathione Sepharose beads for 4 hours at 4°C and analyzed by Western blot technique.

Tet-Off expression and induction of cell cycle arrest. The induction of HTm4 expression was done using the Tet-Off expression system of CLONTECH for the studies of its interaction with KAP, Cdk2, and induced cell cycle arrest. HTm4 proteins in both full-length and C-terminal truncated (without the last 22 amino acids, denoted as HTm4-C) forms were used for side-by-side comparison studies. The maintenance and induction of cell culture were carried out according to manufacturer's protocols. In brief, to generate cell clones expressing the inducible HTm4 constructs we constructed HTm4 and its variants in the pTRE2 vector with an in-frame C-terminal Flag epitope tag. U937 cells expressing the tetracycline-controlled transacti-
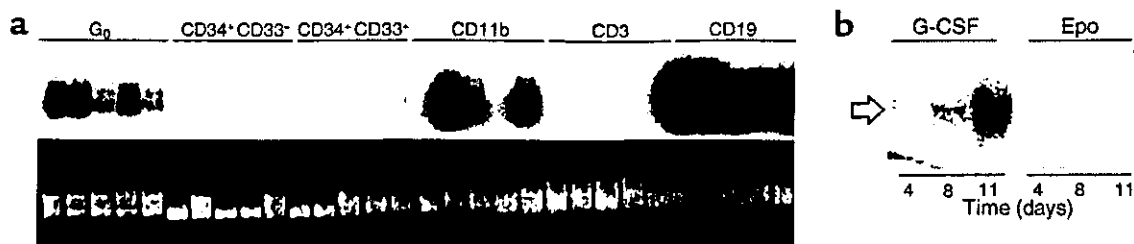
a G₀ CD34⁺CD33⁻ CD34⁺CD33⁺ CD11b CD3 CD19   b G-CSF Epo
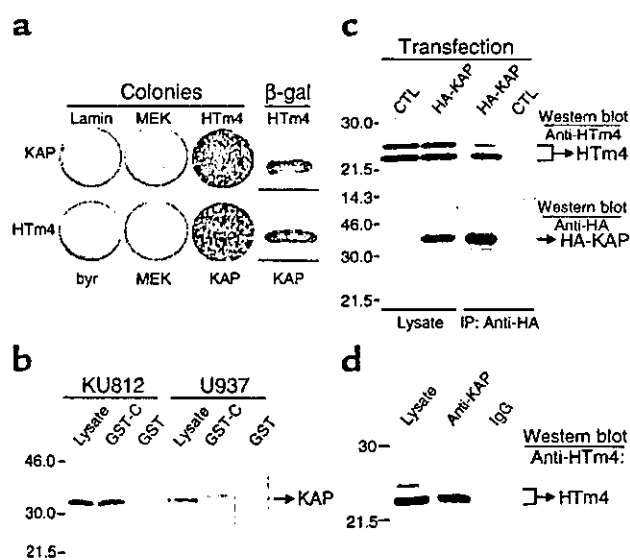
4 8 11   4 8 11
Time (days)

Figure 1

The expression of HTm4 is highly regulated during the differentiation of hematopoietic stem cells. (a) MicroRT-PCR analysis of HTm4 expression in single hematopoietic cells at various stages of differentiation. Cell surface markers for each sample are denoted on the top row. G₀ represents the quiescent CD34⁺/CD38⁻ HSC. The middle row is a Southern blot analysis of HTm4 cDNA obtained through RT-PCR technique. The bottom row shows the loaded cDNA stained with ethidium bromide dye. Each lane represents a single cell. (b) Northern blot analysis of HTm4 expression in CD34⁺ cells that were induced to differentiate with the treatment of either G-CSF or Epo for time intervals as indicated. At day 11, cell populations are determined to be 60% neutrophils and 70% erythrocytes when induced with G-CSF and Epo, respectively.

vator were used as the host. Cells were cotransfected by electroporation with 5 μg of expression vector and 0.25 μg of pHyg plasmid containing the hygromycine resistance gene per 10⁷ cells. Positive clones were maintained in medium containing 200 μg/ml hygromycin and 1 μg/ml of doxycycline (Dox). In the Tet-Off system, the expression of HTm4 and HTm4-C proteins were induced in the absence of Dox, while their suppressions were achieved in the presence of Dox (1 μg/ml). Cells were synchronized as described by Ling et al. (31). Briefly, 10⁶/ml of cells was treated with two rounds of incubation with 2 mM thymidine for 16 hours each, separated by 10 hours of resting period in the absence of thymidine. The first thymidine treatment was done in the presence of 10% FBS and the second in 0.5% FBS. The stimulation of cell growth, after synchronization, was done in the presence of 15% FBS. Cell samples analyzed at the time points indicated were fixed with 70% alcohol at 4°C for 30 minutes, stained with propidium iodide (50 μg/ml), and then analyzed by flow cytometry.

Immunohistochemical studies. All staining was performed by standard immunoperoxidase methods. Briefly, slides were deparaffinized and were either not pretreated (HTm4), pretreated in 1 mM EDTA, pH 8.0, for 20 minutes at 95°C (Ki-67), or pretreated in 10 mM sodium citrate, pH 6.0, for 20 minutes at 95°C (CDK2 and KAP). All further steps were performed at room temperature in a hydrated chamber. Slides were pretreated with Peroxidase Block (DAKO Corp., Carpinteria, California, USA) for 5 minutes to quench endogenous peroxidase activity, and a 1:5 dilution of goat serum in 50 Mm Tris-Cl, pH 7.4, for 20 minutes to block nonspecific binding sites. Either affinity-purified rabbit anti-HTm4 Ab (1:1,000 dilution in 50 mM Tris-Cl, pH 7.4, with 3% goat serum); murine anti-CDK2 Ab (1:500 dilution in 50 mM Tris-Cl, pH 7.4, with 3% goat serum; BD Biosciences, PharMingen-Transduction Laboratories, San Diego, California, USA); murine anti-KAP AB (1:1,000 dilution in 50 mM Tris-Cl, pH 7.4, with 3% goat serum; BD Biosciences, Pharmingen-Transduction Laboratories); or murine anti-Ki-67 Ab

Figure 2

(a) Binding of HTm4 to KAP in yeast two-hybrid assay. Constructs listed in the upper and lower rows were in pGADGH, whereas those to the left of the first column were in pVJLII. HTm4 listed here represents the C_HTm4. MEK, byr, and lamin are negative controls. The positive results are shown as colony growth on selective medium and blue color reaction in the presence of β-gal. (b) Isolation of KAP from U937 and KU812 lysates using GST-C_HTm4 fusion protein. GST-C represents GST-C_HTm4. Lysates derived from U937 and KU812 are absorbed with either GST-C_HTm4 or GST-coupled beads, and bound proteins were analyzed in a Western blot assay for the presence of KAP. (c) Coimmunoprecipitation of HA-tagged KAP and HTm4. CTL, pCMV control vector. The source of the samples is listed on the top. The first two columns on the left are lysates alone and the remaining two are after immunoprecipitation with anti-HA. Samples are analyzed by the Western blot technique using anti-HTm4 for the top two panels and anti-HA for the bottom two. The identified proteins are listed on the right. (d) HTm4 and KAP form a physiological complex. The source of the samples is denoted on the top; the first column from the left is U937 lysate alone; the second and third are after immunoprecipitation with anti-KAP and IgG control, respectively. The presence of HTm4 is indicated on the right after Western blot analysis. Representative figures of at least five experiments.

a

Colonies   β-gal
Lamin MEK HTm4   HTm4
KAP   30.0-
                              21.5-
                              14.3-
HTm4   46.0-
                              30.0-
byr MEK KAP KAP   21.5-

b

KU812   U937
46.0-
30.0-   →KAP
21.5-

c

Transfection
CTL HA-KAP HA-KAP CTL
30.0-   Western blot
21.5-   Anti-HTm4
                           ⊐►HTm4

14.3-   Western blot
46.0-   Anti-HA
30.0-   →HA-KAP
21.5-
Lysate IP: Anti-HA

d

Lysate Anti-KAP IgG
30-   Western blot
                         Anti-HTm4:
21.5-   ⊐►HTm4

(MIB-1 clone; 1:100 dilution in 50 mM Tris-Cl, pH 7.4, with 3% goat serum; Coulter Corp., Miami, Florida, USA) was applied at room temperature for 1 hour. After washing in 50 mM Tris-Cl, pH 7.4, secondary goat anti-rabbit or goat anti-mouse HRP-conjugated Ab (Envision detection kit; DAKO Corp.) was applied for 30 minutes. After further washing, immunoperoxidase staining was developed using a 3,3-diaminobenzidine chromogen kit (DAKO, USA) per the manufacturer and counterstaining was done with hematoxylin.

Results

Yeast two-hybrid and fusion protein assays. Using the yeast two-hybrid assay, we have detected the binding of HTm4 to KAP (Figure 2a). The specificity of this binding has been confirmed by the isolation of KAP from U937 and KU812 lysates, using a fusion protein of GST and the last 25 amino acids of the C-terminal region of HTm4 (Figure 2b).

HTm4-KAP immunoprecipitation. The binding of HTm4 to KAP under physiologic conditions has been established by two different approaches. First, the binding has been detected between the endogenous HTm4 of KU812 and the exogenous HA-tagged KAP after the immunoprecipitation with anti-HA mAb (Figure 2c). Second, to eliminate the possibility that the binding between HTm4 and HA-KAP was spurious, due to the overexpression of the exogenous HA-KAP, we performed a direct immunoprecipitation of endogenous KAP in KU812 cells with anti-KAP Ab (Figure 2d). The resulting immune complexes have been determined to contain the endogenous HTm4, indicating the interaction between KAP and HTm4 is physiologic.

HTm4-KAP Immunohistochemistry. KAP is located predominantly in the perinuclear area (34), and in U937 HTm4 colocalizes with KAP principally in the same region (Figure 3a). This observation substantiates the physiologic interaction between HTm4 and KAP. The subcellular location for HTm4 has been determined to be in the microsomal membrane fraction (Figure 3b). Our anti-HTm4 polyclonal Ab recognizes the native HTm4 as a doublet. The intracellular staining pattern (Figure 3c) is consistent across the many cells types thus far examined.

HTm4-KAP-CDK2 Immunoprecipitation. KAP is known to bind to CDK2 and Cdc2 (17, 18). It is demonstrated here that HTm4 binds to KAP-CDK2 complexes (Figure 4a) in immune complexes derived from U937 lysate using anti-CDK2 mAb. CDK2 is detected as doublets, with the upper band being the dephosphorylated form and the lower the phosphorylated form (Figure 4c). The immunoprecipitated complexes may be composed of solely HTm4-KAP-CDK2 or as a mixture with CDK2-KAP. In light of our previous yeast two-hybrid assay, which showed only the binding between HTm4 and KAP, it is unlikely that HTm4 interacts directly with CDK2. The C-terminus is responsible for the binding of HTm4 to KAP in the yeast two-hybrid assay. Therefore, we examined

whether the C-terminus of HTm4 is required for its binding to KAP-CDK2. As shown in Figure 4b, the C-terminal region indeed is required for the binding of HTm4 to KAP-CDK2 complexes.

CDK2 phosphorylation status. KAP is known to dephosphorylate Thr[160] in the human CDK2 in the absence of cyclin A (16), acting as a negative regulator. It can also bind to CDK2 in the presence of cyclin A; however, the formation of cyclin A-CDK2 complexes does preclude the dephosphorylation of CDK2 by KAP. The phosphatase activity of KAP is enhanced upon the binding of HTm4 as demonstrated in Figure 4c, which shows that the exogenous expression of full-length HTm4 promotes the dephosphorylation of CDK2, whereas, in the presence of C-terminal truncated HTm4, the level of phosphorylated CDK2 remains unchanged. This observation implicates the modulation of CDK2 by HTm4 through its direct binding to KAP.

Induction of cell cycle arrest. We here evaluate the effects of HTm4 on cell cycle progression through the overexpression of intact HTm4 in synchronized U937 cells (Figure 5). After 20 hours of serum stimulation, 60.8% ($n = 5$; the percentage given is an average derived from five experiments) of cells, in the presence of exogenous HTm4, accumulate at G_0/G_1 phase of cell cycle in contrast to 35% ($n = 5$) in the absence of exogenous HTm4. This effect is not detected in U937 cells with exogenous
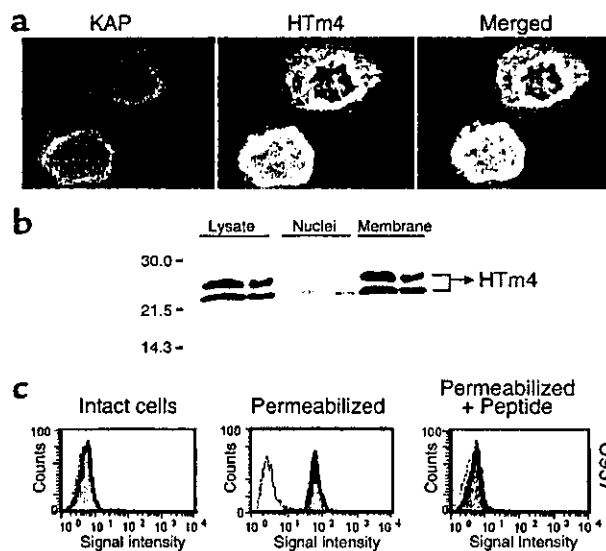

Figure 3

(a) Colocalization of HTm4 and KAP. Staining was visualized with goat anti-mouse IgG Texas Red–conjugated (left) or goat anti-rabbit IgG FITC–conjugated (middle) secondary Ab's. Merged image is given on the right showing that HTm4 and KAP colocalized predominately in the perinuclear area. (b) HTm4 is located in the intracellular membrane fraction. Source of the samples are denoted on the top row. Samples shown are in duplicates. The presence of HTm4 is indicated on the right after Western blot analysis. (c) Staining of U937 with anti-HTm4 Ab. The manipulations of samples are denoted on the top. The filled peaks represent the staining with anti-HTm4, and the open peaks represent IgG control. *x* axis, cell counts; *y* axis, signal intensity of FITC fluorescence. Representative figures of at least five experiments.
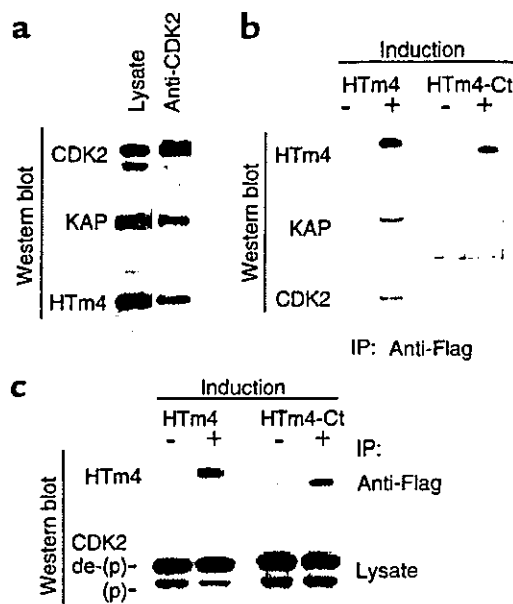
Figure 4
(a) HTm4 binds to KAP-CDK2 under normal physiological conditions. Samples analyzed are shown on the top. The lane marked anti-CDK2 includes the samples immunoprecipitated with anti-CDK2 mAb and analyzed by Western blot technique using Ab's as indicated on the left. (b) The C-terminal region of HTm4 is required for its binding to KAP-CDK2. HTm4-Ct, HTm4 without the last 22 amino acids. Ab's used for the Western blot analysis, after immunoprecipitation with anti-Flag, are listed on the left. The induction of protein expression in the absence of Dox is marked as +, and no induction in the presence of Dox is marked as -. (c) The C-terminal region of HTm4 is required for the enhancement of the phosphatase activity of KAP. The descriptions for c are the same as Figure 4b, except the top panels show the immunoprecipitation with anti-Flag Ab and the bottom panels are derived from 50 μg of total lysate per sample. The upper bands in the lower panels are the dephosphorylated form of CDK2, marked as de-(P), and the lower bands are the phosphorylated (P) form. A reduction in intensity can be seen in the phosphorylated CDK2 in the presence of overexpressed Flag-HTm4. Representative figures of at least five experiments.

C-terminal truncated HTm4, 36.3% versus 34.4% (induced vs. noninduced; n = 5 for both).

Immunohistochemical studies. We then studied the expression of CDK2, KAP, and HTm4 in human tissues using immunostaining with anti-CDK2, anti-KAP, and anti-HTm4 Ab's. Figure 6 shows dense expression of all three proteins in the proliferating cell populations of germinal centers within secondary follicles of human tonsillar tissue. No significant expression of CDK2, KAP, or HTm4 was seen in the surrounding mantle zones of the follicles. All three proteins also demonstrated similar patterns of cellular colocalization with predominantly paranuclear/cytoplasmic staining, and to a lesser degree, nuclear staining, most evident with CDK2. This pattern of expression coincides exactly with cells that are in the active cell cycle, as evidenced by an identical staining pattern seen for Ki-67, a nuclear cell proliferation-associated antigen, expressed in all active stages of the cell cycle

(Figure 6d). Taken together, these findings suggest that these proteins are expressed specifically in actively cycling cells of the germinal centers within secondary follicles of human lymphoid tissues.

Discussion

We have shown that HTm4 is expressed in hematopoietic cells and tissues and highly regulated during the differentiation of hematopoietic stem cells. Our data also indicate that HTm4 expression is regulated during HSC progression through the cell cycle. This regulation seems to be both stage- and lineage-specific. Under certain cellular conditions, the expression of HTm4 is likely to be associated with the exit from cell cycle progression. As presented here, HTm4 is detected in G_0 quiescent stem cells and terminally differentiated hematopoietic cells of certain lineages. The biological role of HTm4 we elucidated is consistent with this observation.

Of particular interest to hematopoietic cell cycle control, we explored the interaction between HTm4 and KAP and the effects on CDK2 phosphorylation and cell cycle progression. Our data indicate a physiologic HTm4-KAP interaction. HTm4 binds to KAP-CDK2 complexes through the binding of KAP to HTm4 and can promote detectably the phosphatase activity of KAP. The C-terminal region of HTm4 is required for these actions. The overexpression of HTm4 can cause cell cycle arrest at G_0/G_1 phase. We thus identify HTm4 as a novel hematopoietic modulator for the G_1-S cell cycle transition. We here put our findings in the context of previous pertinent research.
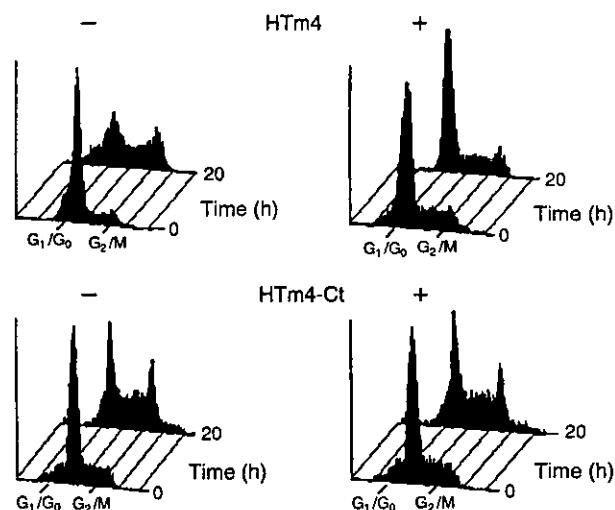

Figure 5
Cell cycle arrest is induced by the exogenous expression of HTm4. The upper panels are U937 with inducible Flag-HTm4 expression vector and the lower panels are with Flag-HTm4-Ct. +, induction of expression in the absence of Dox; -, no induction in the presence of Dox. Time intervals are given on the right. 0 hours, before the addition of FBS into the cultures; 20 hours, cells were cultured in the presence of serum for 20 hours after synchronization. X axis shows cell cycle phase analyzed. Marked here are G_0/G_1 and G_2/M; in between (not marked) is S phase. Representative figures of at least five experiments.
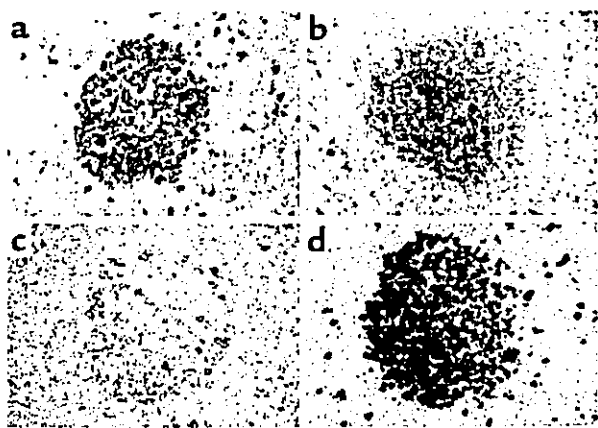
Figure 6
Immunoperoxidase staining of secondary follicles of the tonsil with CDK2, HTm4, KAP, and Ki-67. Sections of reactive tonsillar tissue are stained for expression of (a) CDK2, (b) HTm4, (c) KAP, and (d) Ki-67. Note the strong staining of the proliferating cells of the germinal center for all Ab's with absence of staining of the surrounding mantle zones. The cellular protein localization is predominantly nuclear for CDK2 and Ki-67 (a and d, respectively) with nuclear and paranuclear staining noted for HTm4 and KAP (b and c, respectively). Magnification ×400, HRP stain; hematoxylin was used as counterstain.

Our observations are consistent with the functional role of CDK2, which has been shown to be required for the transition from G_1 to S phase of cell cycle (12). Previous research demonstrated that disruption of CDK2 activity by deactivation or overexpression may cause either G_1 cell cycle arrest (35) or uncontrollable cell growth (36), respectively. Creating a situation similar to the deactivation of CDK2, the exogenous expression of HTm4 causes the progression of cell cycle to slow and therefore implicates a role for HTm4 in cell cycle regulation. There are two possible modes of action, which might work in concert, for HTm4 to accomplish its functional role in vivo. First, the binding of HTm4 to KAP-CDK2 might enhance the phosphatase activity of KAP, which in turn would deactivate CDK2 at a faster rate. CDK2 is known to be such a potent kinase that it can inactivate the exogenously expressed retinoblastoma protein endogenously, and thus, prevent cell cycle arrest (37–39). The fine-tuning of CDK2 kinase activity may be essential for the balanced cell cycle progression. Second, because HTm4 is a transmembrane protein located in the perinuclear area, it might function to sequester KAP-CDK2 or KAP-CDK2-cyclin E/A. This would keep these complexes out of their functional milieu and impact the transition from G_1 to S phase and S phase progression of the cell cycle. Put in the context of previous findings, our data support the biological role of HTm4 as a regulator that modulates and possibly maintains a critical level of cyclin E/A-CDK2 kinase activity during the G_1-S transition and S phase progression.

Our data on cell cycle arrest caused by the overexpression of exogenous HTm4 are consistent with the find-

ings of Yeh et al. (19). We have demonstrated that expression of HTm4 stimulates the phosphatase activity of KAP and possibly sequesters CDK2 from its functional pathway, attenuating cell cycle progression at G_0/G_1. Yeh et al. found that the KAP produced by a hepatoma was defective in its ability to bind CDK2. This would result in a persistently activated CDK2 and uncontrollable cell growth. Interestingly, though, it has been shown that KAP is upregulated in both breast and prostate cancers (34). This apparent contradiction can be explained by the fact that in this study the integrity of KAP and its associated pathway were not confirmed. In addition, it should not be assumed that the same abnormality would yield an identical outcome in different cell contexts, because the status of other key cell cycle regulators may vary (3). When the downstream components are impaired, the resulting phenotypes cannot be overcome even when an upstream antagonist is overexpressed (40). The accompanying upregulation of regulators, along the same pathway as the damaged signaling component, might have been compensatory rather than causative (41).

Interestingly, our confirmation of the in vivo coexpression of KAP, CDK2, and HTm4 in the proliferating cell populations of germinal centers within secondary follicles of human tonsils showed that these proteins are highly expressed in the actively cycling cells. As we have described both in the Discussion and Introduction, the observation that HTm4 is expressed in both quiescent and actively cycling cells has basis and precedent. As described, p21 and p27 have been reported to have dual functions in cell cycle progression (42, 43). The same explanations for expression in proliferating and non-proliferating states that apply to p21 and p27 apply to HTm4: (a) expression of an inhibitory cell cycle regulator in response to proliferation or (b) an effect that is modified by the concentrations of other cell cycle regulators, both known and unknown (3, 42, 43).

In conclusion, our research has provided substantial evidence for HTm4 as a novel hematopoietic modulator for the G_1-S cell-cycle transition. We have identified that HTm4 binds to KAP and that HTm4 forms a physiologic complex with KAP and CDK2. Exogenous expression of HTm4 promotes the dephosphorylation of CDK2, leading to cell cycle arrest at G_0/G_1 phase. The immunohistochemistry data showing expression of HTm4, KAP, and CDK2 in the germinal centers of lymphoid tissues highlight the potential biological relevance of our observations in vivo.

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Genetic Variants of the Receptors for Thromboxane A2 and IL-4 in Atopic Dermatitis

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Thromboxane A2 (TXA2) is an arachidonate metabolite which is considered to relate to chronic inflammation in atopic diseases characterized by elevated immunoglobulin E productivity. The elevation of immunoglobulin E levels involves many molecules including interleukin-4 (IL-4) and interleukin-4 receptor alpha chain (IL-4Rα). To assess whether genetic variants of TXA2 receptor, IL-4 and IL-4Rα genes relate to the elevation of serum immunoglobulin E levels in patients with atopic dermatitis (AD), we conducted an association study of genetic polymorphisms of TXA2 receptor (795C/T), IL-4 (−589C/T), and IL-4Rα (Ile50Val) in a Japanese population ($n = 789$). The TXA2 receptor 795TT genotype strongly related to AD with high serum immunoglobulin E concentrations. AD patients with both TXA2 receptor 795TT genotype and the IL-4Rα Ile50/Ile50 genotype showed the greatest immunoglobulin E concentrations. These results suggest TXA2 receptor polymorphism strongly interacts with IL-4Rα polymorphism as a major determinant of high serum immunoglobulin E levels in AD. © 2002 Elsevier Science (USA)

Key Words: atopic dermatitis; heterogeneity; IL-4 receptor alpha chain; polymorphisms thromboxane A2 receptor.

Atopic dermatitis (AD) is a chronic inflammatory skin disease, which results from the interaction between genetic and environmental factors (1, 2). Although several candidate genes for AD, including interleukin-4 (IL-4) and interleukin-4 receptor alpha chain (IL-4Rα), have been proposed, it is considered that each gene has a relatively small effect to the development of AD (3–5). IL-4 acts through IL-4Rα and activates STAT6 and promotes immunoglobulin E (IgE) production by B cells, and mice deficient in *Il4*, *Il4r*, and *Stat6* lack IgE synthesis and Th2-type reaction (6). Previously we reported the lack of association between AD and the genetic variants of IL-4 and IL-4Rα gene, however, the possibility that these genes cooperate with other genes in modifying the expression of AD, especially in the elevation of IgE productivity, is not excluded (7). It is well known that some patients with AD show very high total serum IgE concentration and several factors including concomitant atopic respiratory disease (8), severity of AD (9), and colonization of the skin with *Staphylococcus aureus* (10) contribute to the elevation of IgE levels in patients with AD. However, the mechanisms underlying the elevated IgE levels of AD patients are still unknown.

Recently Unoki *et al.* have reported a significant association between bronchial asthma and the genetic variant of thromboxane A2 (TXA2) receptor (11). TXA2 is an arachidonate metabolite which is considered to relate to chronic inflammation, and it is a potent stimulator of platelet aggregation and a constrictor of vascular and respiratory smooth muscles (12). TXA2 and its receptor have been implicated as mediators in several diseases such as myocardial infarction, stroke, and bronchial asthma (13). It is well known that coexistence of asthma and AD is common and more than 40% of asthmatics have a history of AD (14). Thus, it is possible that TXA2 and its receptor might relate to chronic inflammation of AD. We therefore conducted a genetic association study to assess the involvement of TXA2 receptor polymorphisms with AD in a Japanese population. We also examined whether the genetic variants of IL-4 and IL-4Rα play any role in the elevation of IgE in patients with AD, interacting with the genetic variants of TXA2 receptor.

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TABLE 1

Association between a Variant of Thromboxane A2 (TXA2) Receptor and Atopic Diseases

| | No. of cases | TXA2 receptor C795T genotype | | | | |
		CC	CT	TT	Odds ratio* (95% CI)	P
Controls	178	30 (17%)	84 (47%)	64 (36%)		
Atopic dermatitis						
Total	360	46 (13%)	175 (48%)	139 (39%)	1.12 (0.77–1.63)	0.549
Very high IgE[b]	89	10 (11%)	27 (30%)	52 (59%)	2.50 (1.49–4.22)	0.0004
Extensive	125	17 (14%)	49 (39%)	59 (47%)	1.59 (1.00–2.54)	0.050
Asthma (+)	112	13 (12%)	54 (48%)	45 (40%)	1.20 (0.74–1.95)	0.470
Rhinitis (+)	152	15 (10%)	74 (49%)	63 (41%)	1.26 (0.81–1.97)	0.307
ASE (+)[c]	269	34 (13%)	133 (49%)	102 (38%)	1.09 (0.73–1.61)	0.674
Atopic asthma	111	14 (12%)	53 (48%)	44 (40%)	1.17 (0.72–1.91)	0.529
Allergic rhinitis	106	17 (16%)	56 (53%)	33 (31%)	0.81 (0.48–1.34)	0.406

* CC + CT vs TT.
[b] IgE >8000 IU/ml.
[c] Allergen-specific IgE against house dust mite.

MATERIALS AND METHODS

Subjects. A total of 394 Japanese patients (237 males and 157 females) with AD were included in the study. They ranged in age from 8 to 66 years (mean, 24 years). The diagnosis was based on the morphological appearance of skin lesions, clinical course, and personal and family histories of atopic diseases. All patients fulfilled the criteria of Hanifin and Rajka (15). One hundred twenty five patients showed extensive AD (>70% of body surface was affected) (8). One hundred eleven asthmatic subjects had specialist physician-diagnosed asthma with: (i) recurrent breathlessness and chest tightness requiring on-going treatment; (ii) physician-documented wheeze; and (iii) documented labile airflow obstruction with variability in serial peak expiratory flow rates >30%. They comprised 55 males and 56 females, ranging in age from 18 to 82 years (mean, 50 years). One hundred and six subjects had specialist-diagnosed allergic rhinitis with seasonal nasal obstruction, rhinorrhoea, and sneezing. They comprised 58 males and 48 females, ranging in age from 5 to 76 years (mean, 30 years). One hundred seventy eight healthy volunteers without personal and family histories of atopic diseases were recruited from the employees of Shiga University of Medical Science Hospital; they comprised 79 males and 99 females, ranging in age from 22 to 55 years (mean, 35 years). None of the asthmatic subjects and rhinitis subjects had a history of AD. There were no heavy smokers (>20 cigarettes/day) among the subjects. All individuals agreed to participate in this study and appropriate informed consent was provided. This study was approved by the Ethics Committee, Shiga University of Medical Science.

DNA assay. Venous blood was taken from all subjects for DNA extraction and IgE measurement. Allergen specific IgE (ASE) against house dust mite was detected by the CAP ELISA system (Pharmacia, Uppsala, Sweden) and the criteria for a positive titre were as used previously (16).

DNA samples were extracted using a commercial kit (IsoQuick, ORCA Research, Inc., Bothell, WA, USA). For detection of the TXA2 receptor 795C/T polymorphism, a PCR in a mixture including 1.5 mmol/L of magnesium chloride was performed in a Perkin Elmer Cetus thermal cycler using a preliminary denaturing at 95°C for 15 min and then 45 cycles of 95°C for 30 s, 58°C for 30 s, and 72°C for 30 s, with Red hot DNA polymerase (AB gene, Surrey, UK). The PCR primers were 5'-GCC CCG CCG CCC GCC CTT TGC AGG TCT TCA T-3' and 5'-CGC AAG TAG ATG AGC AGC TC-3' for 795C/T polymorphism of the TXA2 receptor. The underlined sequence was added

as an elongation of PCR product. PCR products were digested with BsrDI.

For detection of the IL-4 −589C/T and IL-4Rα Ile50Val polymorphisms, the fluorogenic 5' nuclease (TaqMan) assay was carried out as described before (7). Amplification of genomic DNA was performed in 96-well optical reaction plates using PTC-200 thermal cycler (MJ Research, Inc., Watertown, MA, USA). Oligonucleotide PCR primers and double dye labelled probes for IL-4 −589C/T were designed using Primer Express (PE Biosystems). The primers for IL-4 −589C/T were 5'-ACG ACC TGT CCT TCT CAA AAC ACT-3' and 5'-GCA GAA TAA CAG GCA GAC TCT CCT A-3'. The TaqMan probe for IL-4 −589C was 5'-AGA ACA TTG TCC CCC AGT GCT GG-3' labelled with the fluorescent dye VIC, and the probe for −589T was 5'-AGC ACT GGG GAA CAA TGT TCT CCC-3' labelled with FAM. The primers for IL-4Rα Ile50Val were 5'-AGG TGA CCA GCC TAA CCC AG-3' and 5'-TGA AGG AGC CCT TCC ACA G-3', and the probe for Val50 was 5'-CCC ACA CGT GTA TCC CTG AGA ACA AC-3' labelled with VIC and the probe for Ile50 was 5'-AGC CCA CAC GTG TGT CCC TGA G-3' labelled with FAM (17). PCR cocktails including both probes (200 nM each), primers (900 nM each), and 1 × TaqMan PCR Master Mix (PE Biosystems) were set up and were added to each well of the 96-well plate, and the volume made up to 25 μl with H_2O and the plates were sealed with optical caps. PCR amplification was carried out under the following cycling conditions: 2 min at 50°C, 10 min at 95°C, then 40 cycles of 94°C for 15 s, 62°C for 1 min. After cycling, plates were placed into the ABI PRISM 7700 Sequence Detector (PE Biosystems) and analyzed for post-PCR allelic discrimination. All samples could not be utilized for each assay and samples that were not amplified by PCR were excluded.

Statistics. Data entry and contingency table analyses were done with SPSS for Windows versions 9.0 and 10.0. Odds ratio, 95% confidence intervals were estimated and significance values were calculated by Pearson chi-square test. If the number in the column was <10, Fisher's exact method was used.

RESULTS

The genotype frequencies of TXA2 receptor 795C/T polymorphism in control individuals were concordant with Hardy-Weinberg equilibrium; p(C) = 0.40, p(T) = 0.60. As shown in Table 1, there was no significant