Original Article

Tag Polymorphisms at the *A20* (*TNFAIP3*) Locus Are Associated With Lower Gene Expression and Increased Risk of Coronary Artery Disease in Type 2 Diabetes

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A20 or tumor necrosis factor (TNF)-induced protein 3 (*TNFAIP3*) is a negative regulator of nuclear factor-κB (NF-κB). We have investigated whether polymorphisms in this gene are associated with increased atherosclerosis in diabetic patients. Five tag single nucleotide polymorphisms (SNPs) were typed in 479 type 2 diabetic patients from Boston, including 239 coronary artery disease (CAD)-positive case subjects and 240 CAD-negative control subjects. Two tag SNPs (rs5029930 and rs610604) were independently associated with CAD; adjusted odds ratios (ORs) for minor allele carriers were 2.3 (95% CI 1.4–3.8, *P* = 0.001) and 2.0 (1.3–2.9, *P* = 0.0008), respectively. The association with rs610604 was dependent on glycemic control, with ORs of 3.9 among subjects with A1C ≤7.0% and 1.2 for those with A1C >7.0% (*P* for interaction = 0.015). A similar interaction pattern was found among 231 CAD-positive and 332 CAD-negative type 2 diabetic patients from Italy (OR 2.2, *P* = 0.05 vs. OR 0.9, *P* = 0.63 in the low vs. high A1C strata, *P* for interaction = 0.05). Quantitative RT-PCR in blood mononuclear cells from 83 nondiabetic subjects showed that rs610604 and rs5029930 minor allele homozygotes have 30–45% lower levels of *A20* mRNA than major allele homozygotes, and heterozygotes have intermediate levels (*P* = 0.04 and 0.028, respectively). These findings point to variability in the *A20/TNFAIP3* gene as a modulator of CAD risk in type 2 diabetes. This effect is mediated by allelic differences in *A20* expression. *Diabetes* 56:499–505, 2007

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CAD, coronary artery disease; MAF, minor allele frequency; NF-κB, nuclear factor-κB; SNP, single nucleotide polymorphism.

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Patients with type 2 diabetes experience a two- to fourfold increase in the risk of cardiovascular death compared with nondiabetic subjects (1). The increase in cardiovascular mortality is mainly secondary to an acceleration of the atherosclerotic process, which may be fostered by hyperglycemia and other cardiovascular risk factors that are frequently associated with diabetes and insulin resistance, including central obesity, increased levels of small LDL, low HDL cholesterol, and hypertension (2). Although different cellular pathways link these factors to atherosclerosis, many involve induction of the transcription factor nuclear factor-κB (NF-κB) (3,4). Activation of this inflammatory master regulator upregulates the expression of cytokines, chemokines, and leukocyte adhesion molecules as well as other genes that regulate cell proliferation and survival, which may all contribute to the inflammatory changes that accompany the atherosclerotic process (5,6).

A20 or tumor necrosis factor–induced protein 3 (*TNFAIP3*) is a cytoplasmic zinc-finger protein that serves as a negative feedback inhibitor of NF-κB activity through the cooperative activity of its two ubiquitin-editing domains (7–10). Upregulation of A20 is observed in inflammatory states and is generally thought to limit the potentially deleterious effects of sustained NF-κB activation (10,11). The possibility that A20 and reduction in NF-κB activity might modulate risk of atherosclerosis was suggested by the findings of linkage between the *A20* gene region and atherosclerosis in an intercross between atherosclerosis-susceptible and -resistant mice (12). A single nucleotide difference (Glu627Ala), determining decreased A20 activity and less effective termination of NF-κB activation in response to proinflammatory stimuli, is thought to be responsible for this effect (13).

The aim of our study was to determine whether genetic variability at the *A20* (*TNFAIP3*) locus modulates susceptibility to atherosclerosis in humans. We investigated the association between single nucleotide polymorphisms (SNPs) tagging the entire *A20* locus and coronary artery disease (CAD) in type 2 diabetes, a condition characterized by chronic NF-κB activation (14–16). We also evaluated the effect of these variants on *A20* expression in circulating mononuclear cells to investigate potential mechanisms for the effects of *A20* polymorphisms in atherosclerosis.

TABLE 1
Tagging SNPs at the *TNFAIP3* locus

refSNP ID	SeattleSNP ID	Position*	Location	Variation	MAF	SNPs in LD bin
rs583522	UI-3255	138231577	Intron 1	A>G	0.4	rs598493, rs643177, rs582757
rs5029930	UI-4055	138232377	Intron 1	A>C	0.11	rs5029928, rs3757173, rs719149, rs719150
rs5029933	UI-5433	138233755	Intron 1	A>G	0.09	rs5029925, rs5029931, rs5029936, rs5029938, rs5029943, rs5029948
rs610604	UI-12789	138241110	Intron 6	C>A	0.48	rs2307860, rs629953, rs5029940, rs661561
rs636617	RF-19384	138247709	3′ flanking	C>T	0.15	—

MAF, MAF in the Seattle Caucasian panel. *Position in the Human Genome Assembly 124 (build 35). LD, linkage disequilibrium.

RESEARCH DESIGN AND METHODS

Two populations of Caucasian individuals with type 2 diabetes were studied, one from Boston (n = 479) and the other from San Giovanni Rotondo, Italy (n = 563). The study protocol and informed consent procedures were approved by the local research ethic committees. Each population included a group of case subjects with CAD and a group of CAD-negative control subjects. The selection criteria have been previously described (17). In Boston, the CAD-positive case subjects, defined as subjects who had a stenosis >50% in at least one major coronary artery or their main branches, were recruited among type 2 diabetic patients who underwent cardiac catheterization at the Beth Israel Deaconess Medical Center. CAD-negative control subjects were Joslin Clinic patients (the Joslin Clinic serves as the Beth Israel Deaconess Medical Center Diabetes Clinic) aged 55 years or more, who had diabetes for 5 years or more but had a negative cardiovascular history and a normal exercise treadmill test (18). The Italian population consisted of type 2 diabetic patients who resided in the Gargano area in the central eastern region of Italy and attended the Endocrine Unit of the Institute Casa Sollievo della Sofferenza in San Giovanni Rotondo, Italy. Case subjects were patients who had angiographic evidence of stenosis >50% in at least one major coronary artery or their main branches (n = 189) or had a previous acute myocardial infarction without angiographic documentation (n = 42). Control subjects included diabetic patients without cardiovascular symptoms and with normal resting electrocardiogram and exercise treadmill test (n = 282) or with coronary stenosis (at angiography) ≤50% (n = 50) (17).

SNP genotyping. Tag SNPs selected on the basis of the linkage disequilibrium pattern as described below were genotyped in the study subjects by means of PCR followed by single base extension/fluorescence polarization (AcycloPrime-FP SNP Detection System) using a Wallac VICTOR2 Multilabel Plate Reader (Perkin-Elmer, Boston, MA). Genotyping quality was tested by including six blinded duplicate samples in each 96-well assay. The average agreement rate of duplicate samples was >99%. Sequences of the primers and probes used for typing are available from the authors.

Data analysis. Data generated by the SeattleSNP initiative in the European descent group were used to identify tag SNPs capturing all common variants at the *A20* locus. Tag SNPs were determined by means of the LD Select software using an r^2 of 0.90 to define linkage disequilibrium bins (19).

Genotype distributions were tested at each polymorphic locus for departure from Hardy-Weinberg equilibrium. For each polymorphic locus, the association with CAD was evaluated by logistic regression analysis including age, sex, BMI, smoking status, hypertension status, and A1C as covariates. The significance of differences in the association of SNPs with CAD between A1C strata was determined by adding appropriate interaction terms (SNP [0,1] × A1C stratum [0,1]) to the logistic regression model. Analyses were conducted in each population separately and in the two population combined. In the latter analysis, an indicator variable for the study population (Boston versus Italy) was added to the model. The significance of differences in the association of SNPs with CAD between study populations was determined by adding an interaction term (SNP [0,1] × study population [0,1]) to the model. Finally, the presence of significant differences between populations in the interaction between SNPs and A1C strata was evaluated by adding a SNP × A1C stratum × population interaction term. The proportion of variability in CAD outcome explained by the SNPs was estimated by calculating the deviance r^2 (i.e., the proportional decrease in −2 log likelihood generated by the introduction of the SNPs into the logistic regression model). The association between CAD and common (≥0.05) *A20* haplotypes was analyzed using Haplo.score in R software (http://www.biostat.wustl.edu/genetics/geneticssoft/manuals/haploscore/haplo.score.html) (20). This method allows adjustment for covariates (in this case, age, sex, BMI, smoking status, hypertension status, A1C, and study population) and provides a global test of association and haplotype-specific tests.

***A20* expression.** Levels of *A20* mRNA were measured by quantitative, real-time PCR in peripheral blood mononuclear cells from 83 nondiabetic employees of the Joslin Diabetes Center (47% men, mean age 31.3 ± 10.2 years, and BMI 24.7 ± 3.7 kg/m^2). Peripheral blood mononuclear cells were isolated from peripheral blood by density gradient (Vacutainer CPT; Becton Dickinson, Franklin Lakes, NJ). RNA was extracted using the RNeasy kit (Qiagen, Valencia, CA). *A20* mRNA levels relative to β-actin mRNA levels were determined by quantitative RT-PCR using two predeveloped TaqMan Gene Expression Assays from Applied Biosystems (Foster City, CA) according to the manufacturer's instructions in an ABI PRISM 7000 Sequence Detection System. *A20*–to–β-actin mRNA ratios were obtained from the equation $2^{-\Delta CT}$, where ΔCT is the difference in threshold cycles between *A20* and β-actin. The significance of mRNA level differences across genotype groups was estimated by linear regression using log-transformed values to account for the skewed distribution of the *A20*—to–β-actin ratios.

RESULTS

The human *A20* (*TNFAIP3*) gene includes nine exons spanning 16 kb on chromosome 6q23. The entire locus has been sequenced by the SeattleSNP initiative in 23 Caucasian subjects, leading to the identification of 22 SNPs having minor allele frequencies >5% in the study population (http://pga.gs.washington.edu/). We used these data to select five tag SNPs representative of all the others on the basis of a pairwise $r^2 \geq 0.9$ (Table 1). The association between these tags and CAD was evaluated in a population of 479 Caucasian individuals with type 2 diabetes from Boston. A second similar set of patients from Italy was used to replicate significant findings. Salient clinical characteristics of both study populations are described in Table 2.

Genotypes were in Hardy-Weinberg equilibrium at each polymorphic locus. Three of the tagging SNPs (rs583522, rs5029930, and rs610604) were significantly associated with CAD in the Boston population (nominal P = 0.042, 0.001, and 0.003, respectively, adjusted for age, sex, BMI, smoking status, hypertension status, and A1C) (Table 3). For both rs5029930 and rs610604, the association was consistent with a dominant model of inheritance, with minor allele carriers having about twice the odds of cardiovascular disease than major allele homozygotes (Table 3). In the case of rs583522, the increase risk of CAD concerned only heterozygotes (odds ratio [OR] 1.7 [95% CI 1.2–2.6]). The three SNPs, although tagging different linkage disequilibrium bins, showed some degree of disequilibrium with each other (r^2 = 0.67 for rs583522 and rs610604; 0.13 for rs5029930 and rs610604; and 0.08 for rs583522 and rs5029930 in the CAD control subjects). To determine the primary driver(s) of the association with CAD, we conducted a logistic regression analysis applying a backward elimination process to a model that initially included all three tags. Both rs5029930 and rs610604, but not rs583522, were retained in the final model (P = 0.012 and 0.013, respectively), indicating that these two SNPs were independently associated with CAD, whereas the

TABLE 2
Clinical characteristics of CAD-positive case subjects and CAD-negative control subjects with type 2 diabetes from Boston and from Italy

	Boston		Italy	
	CAD$^-$	CAD$^+$	CAD$^-$	CAD$^+$
n	240	239	332	231
Men (%)	57.92	64.44	41.69	66.96
Age (years)	65 ± 7	65 ± 8	60 ± 8	65 ± 8
Age at diabetes diagnosis (years)	52 ± 8	53 ± 10	49 ± 9	50 ± 11
Diabetes duration (years)	12 ± 6	12 ± 9	11 ± 8	15 ± 9
BMI (kg/m^2)	31.8 ± 5	32 ± 6	31.3 ± 5	29.9 ± 5
A1C (%)	7.2 ± 1.2	7.3 ± 1.3	8.5 ± 1.9	8.7 ± 1.9
Treatment				
Diet only	7.26	8.90	13.07	9.17
Oral agents	54.27	46.61	51.37	35.37
Insulin	38.46	44.49	35.56	55.46
Hypertension	75.31	83.47	76.32	85.09
Ever smoked	40.51	71.12	28.88	43.06

Data are % or means ± SD.

effect of rs583522 was secondary to its linkage disequilibrium with rs610604.

Associations between rs5029930 and rs610604 and CAD were weaker and did not reach statistical significance in the Italian population (Table 4). The ORs for minor allele carriers were 1.4 for rs5029930 and 1.1 for rs610604, and the 95% CIs included one in both cases. We hypothesized that such lack of replication might be due to differences in some of the clinical characteristics of the two populations. The most prominent disparity was in the degree of glycemic control, which was significantly better in Boston than in the Italian population (mean A1C 7.3% in Boston vs. 8.6% in Italy, $P < 0.0001$) (Table 1). In a stratified analysis of the Boston population by median A1C value (7.0%), it became clear that most of the association between SNPs and CAD observed in this group originated from the lower A1C stratum. For instance, the OR of CAD for rs610604 minor allele carriers was 3.9 in subjects with A1C ≤7.0% compared with 1.2 in subjects with A1C >7.0% (P for interaction = 0.015) (Table 5). A similar interaction between rs610604 and A1C was observed in the Italian subjects (OR 2.2 for those with A1C ≤7.0 vs. 0.9% in the A1C >7.0% group, P for interaction = 0.05). Thus, the weaker association with CAD observed in the Italian group before stratification was explained by the smaller proportion of individuals with A1C ≤7.0% in this population (22%) compared with that from Boston (49%) (Table 5). If data were analyzed by A1C stratum, the ORs of CAD were not significantly different in Boston and Italy, and a significant association ($P = 0.05$) was also detected in the Italian population in the good glycemic control stratum (Table 5). In the two populations combined, the minor alleles of both SNPs were associated with a threefold increase in the risk of CAD among individuals with low A1C ($P = 0.0012$ and 8.3×10^{-6} for rs5029930 and rs610604, respectively). There was no significant effect in the high A1C stratum (Table 5). Together, the two SNPs explained ~6% of the variability in CAD outcome in the good glycemic control stratum (deviance $r^2 = 0.062$) compared with only 0.4% in the poor glycemic control stratum. A similar interaction between SNPs and glycemic control was observed if A1C values of 6.5 or 7.5% rather than 7.0% were used to stratify the study populations (data not shown).

TABLE 3
Genotype frequencies of *TNFAIP3* tag SNPs in the Boston population according to CAD status

	CAD$^-$	CAD$^+$	*P* value*	OR (95% CI)*
n	240	239		
rs583522				
A/A	0.554	0.435		1.0
A/G	0.367	0.502		1.7 (1.1–2.5)
G/G	0.079	0.063	0.042	1.1 (0.5–2.3)
rs5029930				
A/A	0.850	0.753		1.0
A/C + CC†	0.150	0.247	0.001	2.3 (1.4–3.8)
rs5029933				
A/A	0.900	0.858		1.0
A/G + G/G†	0.100	0.142	0.07	1.8 (0.9–3.2)
rs610604				
A/A	0.525	0.368		1.0
A/C	0.392	0.511		1.9 (1.3–2.9)
C/C	0.083	0.121	0.003‡	2.3 (1.1–4.5)
rs636617				
C/C	0.658	0.707		1.0
C/T	0.317	0.276		0.7 (0.5–1.1)
T/T	0.025	0.017	0.34	0.7 (0.2–3.0)

*Adjusted for age, sex, BMI, smoking status, hypertension status, and A1C. †Minor allele homozygotes had expected counts <5. ‡P = 0.0008 for C/C + A/C vs. A/A.

TABLE 4
Genotype frequencies of *TNFAIP3* tag SNPs in the population from Italy according to CAD status

	CAD$^-$	CAD$^+$	*P* value*	OR (95% CI)*
n	332	231		
rs5029930				
A/A	0.883	0.857		1.0
A/C + CC†	0.117	0.143	0.19	1.4 (0.9–2.2)
rs610604				
A/A	0.509	0.502		1.0
A/C	0.419	0.424		1.1 (0.7–1.6)
C/C	0.072	0.074	0.93	1.1 (0.5–2.3)

*Adjusted for age, sex, BMI, smoking status, hypertension status, and A1C. †Minor allele homozygotes had expected counts <5.

TABLE 5
Risk of CAD associated with *TNFAIP3* SNPs according to A1C and study population

	Boston		Italy		Boston +Italy	
	A1C ≤7.0%	A1C >7.0%	A1C ≤7.0%	A1C >7.0%	A1C ≤7.0%	A1C >7.0%
n	229	238	124	426	353	664
rs5029930						
C/C + A/C vs. A/A						
OR (95% CI)	3.32 (1.4–7.7)	1.7 (0.9–3.3)	2.44 (0.8–7.2)	1.20 (0.7–2.0)	2.90 (1.5–5.6)*	1.40 (0.9–2.1)*
P value	0.006	0.096	0.129	0.53	0.0012	0.098
rs610604						
C/C + A/C vs. A/A						
OR (95% CI)	3.87 (2.0–7.3)	1.24 (0.7–2.1)†	2.20 (1.0–4.9)	0.90 (0.6–1.4)‡	3.00 (1.8–4.9)*	1.05 (0.8–1.5)*§¶
P value	3.3×10^{-5}	0.43	0.053	0.63	8.3×10^{-6}	0.83

Data are OR (95% CI). Twenty-five subjects (12 from Boston and 13 from Italy) for whom A1C values were not available were excluded from the analysis. All ORs are adjusted for age, sex, BMI, smoking status, and hypertension status. The ORs for Boston + Italy are also adjusted for study population. *NS for difference in ORs between populations within A1C strata. †$P = 0.015$ for the difference in ORs between A1C strata in the Boston population. ‡$P = 0.05$ for the difference in ORs between A1C strata in the Italian population. §$P = 0.0008$ for the difference in ORs between A1C strata in the two populations combined. ¶NS for difference between populations in the interaction between SNP and A1C strata.

The two SNPs defined four haplotypes, the frequencies of which are shown in Table 6 along with their score statistics for association with CAD in the combined population. In the stratum with A1C ≤7%, the haplotype formed by the two major alleles (haplotype AA) was significantly more frequent in control subjects than case subjects ($P = 4.8 \times 10^{-7}$). Conversely, the three haplotypes carrying at least one of the minor alleles at rs5029930 or rs610604 (i.e., haplotypes AC, CA, or CC) were all more frequent in case subjects than control subjects, with *P* values ranging from 0.001 to 0.006. Associations were not seen between haplotypes and CAD in subjects with A1C >7% (Table 6).

None of the SNPs in the linkage disequilibrium bins tagged by rs5029930 or rs610604 encoded changes in A20 amino acid sequence. Thus, we hypothesized that the association between these two SNPs and CAD risk could be due to allelic differences in *A20* expression. To test this hypothesis, we measured *A20* mRNA levels by real-time, quantitative PCR in peripheral blood mononuclear cells from 83 healthy nondiabetic subjects (47% men, age 31 ± 10 years, and percent ideal body wt 112 ± 16). We chose to study nondiabetic individuals to avoid the potential confounding effects of differences in glycemic control and associated treatments that might have anti-inflammatory effects (e.g., thiazolidinediones). We used two different assays developed by Applied Biosystems, one targeted to exons 2 and 3 and capturing the isoforms corresponding to GenBank entries M59465, BC064689, and BC041790 and the other targeted to exons 1 and 2 and capturing only isoforms M59465 and BC064689 (Fig. 1*A*). With the first assay, minor allele homozygotes at rs5029930 and rs610604 had 45 and 30% lower *A20* mRNA levels, respectively, compared with major allele homozygotes, with heterozygotes having intermediate values (*P* for trend = 0.028 and $P = 0.04$, respectively) (Fig. 1*B*). A similar pattern of association was observed with the second assay, although in this case, significance was reached only for rs610604 ($P = 0.042$) (Fig. 1*C*). A significant association between genotypes and mRNA levels was also observed if data were analyzed according to haplotype carrier status (AA homozygotes vs. AA heterozygotes versus carriers of any other genotype, $P = 0.018$ and 0.038 for the first and second assay, respectively).

DISCUSSION

A sequence variant in the *A20* gene has been previously shown to account for differences in susceptibility to atherosclerosis between mouse strains (12,13). Our results indicate that genetic variants at the *A20* locus have a similar effect on atherosclerosis in humans with type 2 diabetes. Whereas the effect in animals is due to an amino acid substitution (13), the effect in humans is mediated by variants affecting the expression of the *A20* gene, with lower mRNA levels being associated with increased cardiovascular risk and higher levels with protection. We speculate that lower *A20* expression may predispose to CAD by permitting NF-κB to remain more active, with resultant increases in the expression of downstream atherogenic mediators. NF-κB targets have been implicated at many stages in the atherogenic process, including

TABLE 6
Haplotype frequencies in CAD^+ case subjects and CAD^- control subjects in the two populations combined

rs5029930/ rs610604 haplotypes	A1C ≤7.0% (*n* = 353)				A1C >7.0% (*n* = 664)			
	CAD^-	CAD^+	Haploscore*	*P* value†	CAD^-	CAD^+	Haploscore*	*P* value‡
AA	0.726	0.542	−5.034	4.8×10^{-7}	0.664	0.657	−0.671	0.51
AC	0.214	0.327	2.760	0.0058	0.231	0.219	−0.205	0.82
CA	0.014	0.061	3.140	0.0017	0.045	0.044	0.700	0.48
CC	0.046	0.070	3.280	0.0010	0.060	0.079	1.142	0.25

Twenty-five subjects (12 from Boston and 13 from Italy) for whom A1C values were not available were excluded from the analysis. *Haplotype-specific score for association with CAD computed by the Haplo.score program. Positive values denote association with increased risk, negative values with decreased risk. †Global $P = 1.8 \times 10^{-6}$. ‡Global $P = 0.67$.

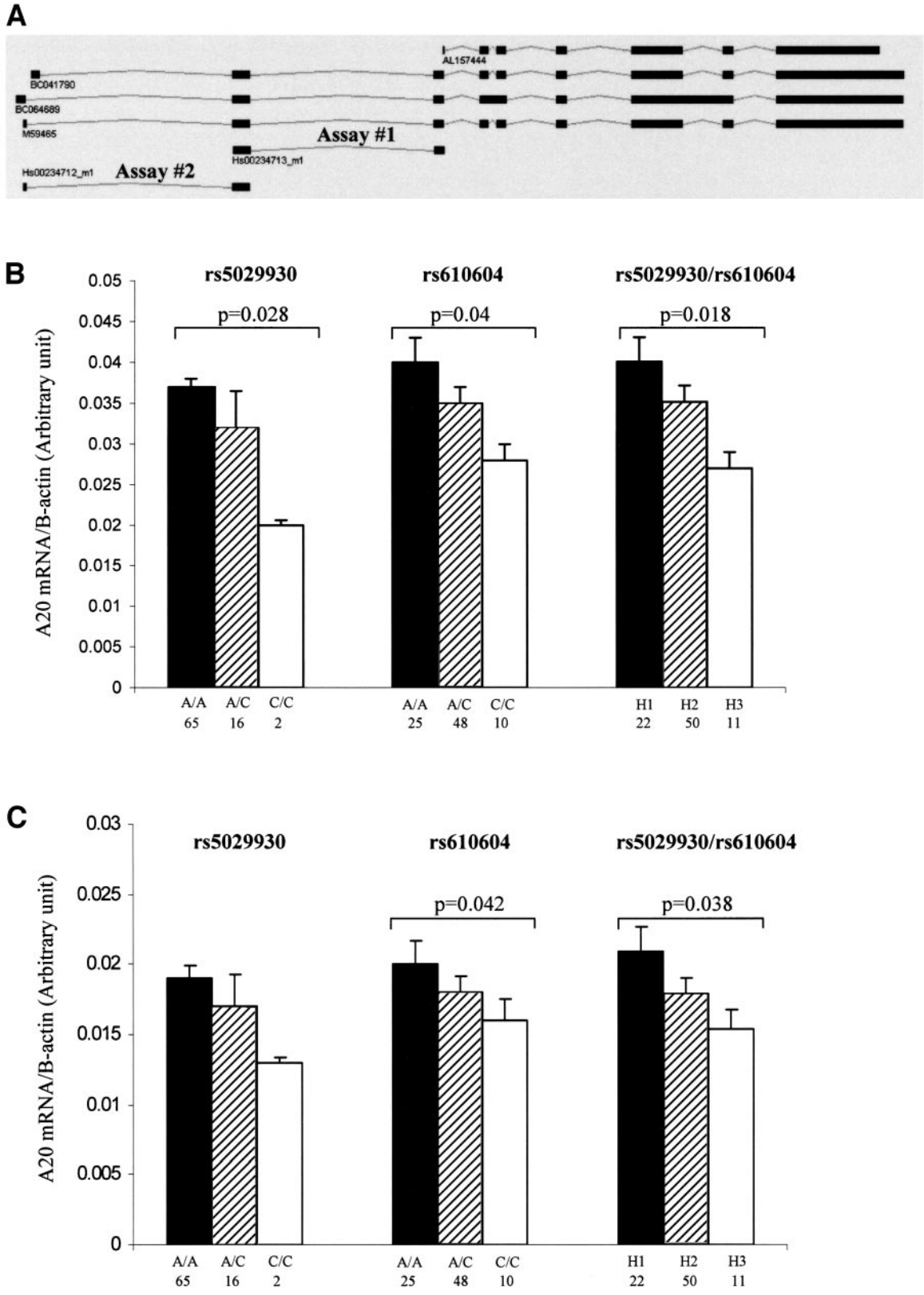

FIG. 1. *A20* mRNA levels in peripheral blood mononuclear cells according to tag SNP genotypes. *A*: Schematic representation of the *A20* gene showing the transcripts measured by each assay. Exons are indicated by black boxes; GenBank accession numbers are indicated below each transcript. *B*: *A20* mRNA levels measured by assay 1 according to rs5029930 and rs610604 genotypes and the diplotypes defined by the two SNPs (H1, AA/AA; H2, AA/X; H3, X/X, where X is any haplotype other than AA). *C*: *A20* mRNA levels measured by assay 2 according to rs5029930 and rs610604 genotypes and the diplotypes defined by the two SNPs (the diplotype notation is the same as in *B*).

P- and E-selectins and intracellular adhesion molecule–1 and vascular cell adhesion molecule–1, which localize circulating immune cells to the endothelium (5,6). Cytokines and chemokines produced locally in atherosclerotic lesions include MCP-1, macrophage inflammatory proteins (MIP-1α, MIP-1β, MIP-2, and MIP-3α), interferon-γ, lymphotoxin, TNF-α, and interleukin-6 (21). Even a small upregulation in NF-κB activity as might be induced by variants decreasing A20 activity would potentially increase the expression of these targets. Conversely, slightly lower NF-κB activity as presumably occurs in carriers of variants increasing A20 activity might decrease the expression of multiple target genes. It is easy to envision how the orchestrated regulation of numerous gene products could translate into clinically apparent phenotypes.

Other studies have examined genes involved in inflammatory processes as candidate loci for CAD. Among the most notable findings to date is the identification of a haplotype in the HLA region on 6p21 that is associated with myocardial infarction in Japanese and Europeans (22,23). Two of the three genes encompassed by this haplotype, *LTA* and *NFKBIL1*, code for modulators of NF-κB, as does the *A20/TNFAIP3* gene. *LTA* encodes lymphotoxin-α, which stimulates NF-κB through its interaction with TNF receptors, whereas *NFKBIL1* encodes a protein homologous to the NF-κB inhibitor, IκB (24,25). Thus, the variants that we identified in the *A20* gene as being associated with CAD could well be part of a larger group of polymorphisms influencing susceptibility to atherosclerosis through a modulation of NF-κB activity.

The lack of significant association in the Italian study before stratification by A1C levels may raise the concern of a false-positive result in the Boston population. On the other hand, the fact that we could replicate the interaction between SNPs and degree of glycemic control in the Italian population argues in favor of a genuine effect of this locus on cardiovascular risk, even if this is limited to a subset of type 2 diabetic patients. Importantly, the same alleles that confer increased cardiovascular risk were found to be associated with decreased levels of *A20* expression in vivo, reinforcing the evidence from the case-control study and providing a plausible biological explanation of the association with CAD.

We do not know why the genetic effect at the *A20* locus is more visible in diabetic individuals under better glycemic control, but we envision two possible scenarios. One is that allelic differences in the inhibitory effect of *A20* may be overpowered by a massive induction of NF-κB secondary to chronically severe hyperglycemia (26). Under these conditions, whether a person has 30–40% lower A20 expression compared with another may be irrelevant. The modulatory effect of allelic differences in *A20* expression may instead be critical in the presence of a more moderate induction of NF-κB such as that elicited by lower (albeit abnormal) glycemic levels. The other possible scenario is that the cellular pathways mediating the increased risk of CAD associated with type 2 diabetes differ in the two glycemic strata. NF-κB–mediated mechanisms may be preponderant in the presence of moderate hyperglycemia, whereas other pathways (e.g., those secondary to PKC activation [4]), may prevail in the presence of more severe hyperglycemia. Under these circumstances, the effect of allelic differences in NF-κB inhibition may be more visible among individuals with relatively good glycemic control than in those with higher glycemic levels.

Two distinct polymorphisms at the *A20* locus were independently associated with CAD in the logistic regression analysis. When the two SNPs were considered together as haplotypes, the two major alleles identified a haplotype that was highly protective, whereas all the three other haplotypes were associated with similar predisposition to CAD. One possible interpretation of these findings is that there are two independent genetic effects at this locus, one tagged by rs5029930, the other by rs610604. For both effects, the minor allele is associated with predisposition to CAD, the major allele with protection. The presence of a minor allele at one locus is sufficient to confer predisposition, and the addition of a minor allele at the other locus does not further increase the risk. Another possibility is that there is a single genetic effect due to an unidentified polymorphism having the major allele in linkage disequilibrium with the protective haplotype and the minor allele in linkage disequilibrium with the three predisposing haplotypes.

As far as the nature of the causal polymorphism(s) is concerned, both rs5029930 and rs610604 are within introns, as are the eight SNPs in the corresponding linkage disequilibrium bins. One of these (rs5029928, in the linkage disequilibrium bin of rs5029930) is in a conserved region, where it disrupts potential binding sites for the transcription factors forkhead box I1 and cut-like homeobox. Whether this affects the transcriptional activation of the *A20* gene will need to be tested further. SNP rs5029940 is also interesting, consisting of a CCT insertion/deletion in intron 2 at a short distance from exon 3. It must be considered, however, that in the HapMap database, linkage disequilibrium with rs5029930 and rs610604 extends, although in a discontinuous fashion, for ~50 kb on both sides of the gene (www.hapmap.org). Thus, the functional variants responsible for the observed associations might be in regulatory elements at some distance from the coding sequence.

There are limitations in our study. We based our SNP selection on the pattern of linkage disequilibrium determined by the SeattleSNP initiative through the resequencing of the DNA from 23 Caucasian subjects (27). This sample size had >99% power to detect SNPs with a minor allele frequency (MAF) ≥10% but only 90% power to detect SNPs with an MAF ≥5%, resulting in the possible exclusion of some SNPs with MAF between 5 and 10% from the linkage disequilibrium analysis. Also, some of the r^2 estimates obtained in this small sample of individuals might have been relatively imprecise, decreasing tagging efficiency. Thus, our study might not have been as comprehensive as one would assume on the basis of our SNP selection strategy. Another potential limitation concerns the selection of CAD control subjects. Because the vast majority of the subjects referred to the catheterization laboratory have significant CAD, we recruited the control group from diabetes clinics on the basis of a negative cardiovascular history, normal resting electrocardiogram, and a normal exercise treadmill test. It is possible that some of the individuals in this group actually had asymptomatic CAD that was missed by the exercise tolerance tests (28). Such misclassification, however, would have biased the results toward the null hypothesis, making our findings of association more notable. Finally, it should be emphasized that these results apply to type 2 diabetes, a condition characterized by insulin resistance and increased propensity to chronic, low-grade inflammation, even in the presence of relatively good glycemic control (15). Whether *A20* polymorphisms influence CAD risk in

the general population will have to be determined by future studies.

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