Abstract. Although a number of environmental risk factors for atherosclerosis have been identified, heredity seems to be a significant independent risk factor. The aim of our study was to identify novel susceptibility genes for atherosclerosis. The screening process consisted of three steps. First, expression profiles of macrophages from subjects with atherosclerosis were compared to macrophages from control subjects. Secondly, the subjects were genotyped for promoter region polymorphisms in genes with altered gene expression. Thirdly, a population of subjects with coronary heart disease and control subjects were genotyped to test for an association with identified polymorphisms that affected gene expression. Twenty-seven genes were differentially expressed in both macrophages and foam cells from subjects with atherosclerosis. Three of these genes, IRS2, CD86 and SLC11A1 were selected for further analysis. Foam cells from subjects homozygous for the C allele at the -765C>T SNP located in the promoter region of IRS2 had increased gene expression compared to foam cells from subjects with the nonCC genotype. Also, macrophages and foam cells from subjects homozygous for allele 2 at a repeat element in the promoter region of SLC11A1 had increased gene expression compared to macrophages and foam cells from subjects with the non22 genotype. Genotyping of 512 pairs of subjects with coronary heart disease (CHD) and matched controls revealed that subjects homozygous for C of the IRS2 SNP had an increased risk for CHD; odds ratio 1.43, p=0.010. Immunohistochemical staining of human carotid plaques showed that IRS2 expression was localised to macrophages and endothelial cells in vivo. Our method provides a reliable approach for identifying susceptibility genes for atherosclerosis, and we conclude that elevated IRS2 gene expression in macrophages may be associated with an increased risk of CHD.

Introduction

Family history seems to be the most important independent risk factor for the development of atherosclerosis (1). Although several Mendelian disorders, such as familial hypercholesterolemia, are known to cause atherosclerosis, they have a low prevalence and only account for a small fraction of the reported incidences (2). It is plausible to assume that multiple genes contribute to the development of atherosclerosis by affecting an individual's response to risk factors. Furthermore, risk factors such as high blood pressure and high cholesterol levels in themselves have a complex genetic influence (1). It is therefore difficult to predict disease progress and to choose an optimal therapeutic strategy in individual patients (3).

Identification of susceptibility genes for atherosclerosis, each with only a limited individual contribution to the disease development, poses a great challenge. It has been proposed that much of the genetic susceptibility to complex diseases is a result of single nucleotide polymorphisms (SNPs) that influence gene expression, and it is estimated that ~6% of all human genes have functional regulatory variants (4). Thus, analysis of variations in gene expression could potentially identify susceptibility genes for complex diseases, which opens the possibility for global expression analysis to identify such genes.

The aim of the study was to search for susceptibility genes for atherosclerosis. We designed a multi-step procedure based on a clinical and population-based sample of subjects with acute coronary syndrome, their first-degree relatives, and a matched control group from the general population called the INTERGENE population study. In the first step, expression profiles of macrophages from subjects with atherosclerosis were compared to macrophages from control subjects to identify genes with an altered gene expression. In the second
step, the association between altered gene expression and the genotype of promoter region polymorphisms was tested. In the third step, the association between the genotype of these polymorphisms and coronary heart disease (CHD) was examined.

We found that insulin receptor substrate 2 (IRS2) was expressed at higher levels in macrophages from subjects with atherosclerosis than in macrophages from control subjects. A SNP in the promoter region of IRS2 was associated with higher gene expression in macrophages, and subjects homozygous for the C allele of this SNP had an increased risk of CHD. Our results indicate that IRS2 might be a susceptibility gene for atherosclerosis and CHD.

Materials and methods

Study outline. Subjects with atherosclerosis and heredity for CHD and control subjects with no atherosclerosis and no family history of CHD were identified from the INTERGENE population study (details below). Using DNA microarray analysis, expression profiles of macrophages and foam cells were generated from these subjects and compared to identify genes with an altered gene expression. The study subjects were genotyped for known polymorphisms in the promoter regions of genes identified by gene expression analysis. Polymorphisms affecting gene expression in macrophages from subjects with atherosclerosis compared to control subjects were then genotyped in a case-control cohort of the INTERGENE study (n=1226). This procedure is outlined in Fig. 1.

Subject. The INTERGENE study was designed to investigate the interplay between genetic, environmental and lifestyle risk factors on the total risk of CHD (http://www2.sahlgrenska.gu.se/intergene/eng/index.jsp). At the time of this study, it was comprised of 670 patients with acute coronary syndromes (myocardial infarction or unstable angina pectoris), 400 first-degree relatives of the patients (siblings, children or parents), and 3600 randomly selected healthy controls from western Sweden (5). An age- and sex-matched case control subpopulation from the INTERGENE study consisting of 613 patients with CHD and 613 non-related controls from the INTERGENE study was used for genotyping studies. The participants gave informed consent, and the ethics committee of Göteborg University approved this study.

The Macrophage INTERGENE study is a subset of the INTERGENE study and was designed to search for potential atherosclerosis susceptibility genes (6). It consists of 15 subjects with atherosclerosis (inclusion criteria: first-degree relatives of the patient group and at least one atherosclerotic plaque in the carotid or femoral artery as assessed by ultrasound) and 15 age- and sex-matched healthy controls without family history of atherosclerotic disease (6). The clinical characteristics of these subjects were described previously (7).

Carotid atherosclerotic plaques were obtained as endarterectomy specimens from four Caucasian patients operated on for symptomatic carotid artery stenosis at Sahlgrenska University Hospital within the framework of the Göteborg Atheroma Study Group (http://www.wlab.gu.se/GASG).

Macrophage preparation. Monocytes from the participants of the Macrophage INTERGENE study were differentiated as described previously (8). Briefly, white blood cells were isolated from buffy coats, seeded and allowed to adhere for 1 h. Non-adherent cells were removed by three washes with PBS, and adherent cells were then allowed to differentiate in Macrophage-SFM (Gibco BRL, Grand Island, NY) for 6 days.

LDL preparation and oxidation were performed as described previously (8). Briefly, human LDL (d 1.019-1.063 g/ml) was prepared by sequential ultracentrifugation at 4°C of plasma from healthy, fasted male volunteers. The LDL was oxidized by storage at 4°C for 3 months to generate minimally modified LDL (mmLDL). The thiobarbituric acid reactive substance (TBARS) levels of the mmLDL were 5 nmol malondialdehyde equivalents/mg protein. On day 7, the macrophages were cultured with mmLDL (50 μg protein/ml) for 24 h to generate lipid-loaded macrophages, as a model for foam cells. The presence of foam cells is considered a hallmark of atherogenesis (8). The macrophages and foam cells were washed in PBS, and RNA was isolated using the RNeasy Kit (Qiagen, Hilden, Germany).

DNA microarray. Biotin-labeled target cRNA was hybridized to individual HG-U133A DNA microarrays and processed according to the manufacturer’s protocol (Affymetrix, Santa Clara, CA). DNA microarrays were scanned using the G2500A GeneArray scanner (Hewlett Packard, Palo Alto,
tide gives TCGCGG, which is not recognized by
in the restriction site CCGCGG, whereas a thymine nucleo-
GCT-3’. The PCR products were incubated overnight with
as starting material and primers forward 5'-CCTGGGTGG
was used. PCR was performed using 20-30 ng genomic DNA
of IRS2, a restriction fragment length polymorphism assay
was genotyped for the two most prevalent alleles of this repeat
CA), and the output files were analyzed with Microarray
suite 5 (Affymetrix). DNA microarrays were globally scaled
to an average intensity of 100. On average, 37% of the probe
sets were classified as present. In all, four groups with 15
expression profiles in each group were generated, and macro-
phages and foam cells from subjects with atherosclerosis
were compared to macrophages and foam cells from control
subjects, respectively. Only genes with an average intensity
signal >50 in at least one group (atherosclerosis or control
subjects, macrophages or foam cells) were included. Genes
that were upregulated in one group and downregulated in the
other group were excluded.

The microarray data set can be viewed in its entirety at
the GEO Accession viewer at the NIH homepage (http://

Real-time RT-PCR. This procedure was performed as
described (9). Briefly, reagents (TaqMan® Reverse
Transcriptase reagents and TaqMan® Universal PCR Master
mix) were purchased from Applied Biosystems (Foster City,
CA). Assay-on-demand probes and primers for IRS2
(Hs00275843_a1) and pre-developed assay reagents for
endogenous control human PPIA, 4333763T, were obtained
from Applied Biosystems.

Genotyping. DNA was isolated from blood samples from
the case control subpopulation in the INTERGENE study. For
the genotyping of the -765C>T SNP in the promoter region
of IRS2, a restriction fragment length polymorphism assay
was used. PCR was performed using 20-30 ng genomic DNA
as starting material and primers forward 5’-CCTGGGTGG
CATCTCCTC-3’ and reverse 5’-GCTGCTGTTGCTGCT
GCT-3’. The PCR products were incubated overnight with
restriction enzyme BglI. A cytosine nucleotide at -765 results
in the restriction site CCGGG, whereas a thymine nucleo-
tide gives TCGGG, which is not recognized by BglI.

Fragment analysis was used for genotyping of the repeat
element of SLC11A1. A PCR product was generated from
20-30 ng genomic DNA using primers forward 5’-GCTTGG
GAACTCCAGATCAAAG-3’ and reverse 5’-TGATATT
CATGTCAATACC-3’. PCR products were size-separated
on an ABI PRISM 3100 genetic analyzer (Applied Biosystems)
based on the number of GT-repeats in the promoter region.

TaqMan-based SNP analysis for genotyping of the
rs2715267 SNP in the promoter region of CD86 was
conducted using Assay-on-demand C_26193522_10 (Applied
Biosystems) according to the manufacturer's protocol.

Immunohistochemistry. Carotid endarterectomy specimens
isolated from 4 patients were divided into 3-mm sections,
fixed in formalin for 24 h and embedded in paraffin. Sections
(4-μm) were taken from each block for immunohisto-
chemistry. The sections were dewaxed, and antigens were
unmasked with target retrieval solution (Dako Cytomation,
Buckinghamshire, UK). Immunohistochemical staining was
performed with a standard LSAB® System-HRP Kit (Dako)
and with primary antibodies directed against macrophage
marker CD68 (Novocastra Laboratories, Newcastle, UK) and
IRS2 (Santa Cruz Biotechnology, Europe). Sections were
incubated with primary antibody overnight. Staining was
performed according to the manufacturer's instructions. As a
negative control, the primary antibody was substituted with a
universal negative control reagent (Dako).

Statistical analysis. All the genes present on the DNA
microarrays were compared, and the Student’s t-test was used
to identify genes that were differentially expressed in macro-
phages and foam cells from subjects with atherosclerosis
compared to the controls. P<0.05 (two-tailed) was considered
significant. The Kruskal Wallis test was used to test for
association between genotype and gene expression.

For real-time RT-PCR, the unpaired Wilcoxon test was
used to test for difference in gene expression between
macrophages from subjects with atherosclerosis and control
subjects, and the paired Wilcoxon test was used to test for
differences in gene expression between macrophages and
foam cells from the same individual. Results were expressed
as the mean ± SEM. The Mantel-Haenszel common odds ratio
estimate and the McNemar’s test were used to test for the
association between CHD and the genotype in matched case-
control pairs, analyzing the occurrence of discordant pairs.

Results

Expression profiling. Twenty-seven genes had a significantly
altered gene expression in macrophages and foam cells from
subjects with atherosclerosis compared to the controls (Table I).
Gene expression of 7 of the 27 regulated genes was also
significantly affected in foam cell formation in both the
atherosclerosis and control group. Among the identified
genes were both genes that were previously described in the
context of atherosclerosis, insulin receptor substrate 2 (IRS2)
(11,12) and CD86 (10), as well as several novel findings,
such as SLC11A1 and KIAA1522. One of the genes with an
altered gene expression, CD44, was studied in a previous
article (6).

Three genes were selected for validation as potential
susceptibility genes for atherosclerosis. IRS2 and CD86 were
chosen since both genes were previously shown to affect the
development of atherosclerosis in mice (10,11). SLC11A1,
also known as NRAMP1, was chosen since it is located in a
chromosomal region previously linked to CHD (12).

Association between genotype and gene expression. A
promoter region SNP, -765C>T, was previously shown to
affect IRS2 gene expression (13). This SNP was genotyped
in the Macrophage INTERGENE study. Foam cells from
subjects homozygous for C, CC genotype, had higher IRS2
gene expression in both macrophages and foam cells from
subjects homozygous for allele 2, 22
P=0.044 (Fig. 2A).

A promoter region repeat element of SLC11A1 was
previously shown to affect gene expression (14). The
participants of the Macrophage INTERGENE study were
genotyped for the two most prevalent alleles of this repeat
element, allele 2 and 3. Subjects homozygous for allele 2, 22
genotype, had higher gene expression in both macrophages
(P=0.009) and foam cells (P=0.015) than subjects with the 23
and 33 genotype (Fig. 2B).

The SNP in the promoter region of CD86, -3479G>T
rs2715267, was shown to affect binding affinity for nuclear
proteins, where the G allele was associated with decreased affinity (17). However, when this SNP was genotyped in the Macrophage INTERGENE study, no differences in gene expression were observed (data not shown).

Genotype association with coronary heart disease. The promoter region polymorphisms of IRS2 and SLC11A1 were also tested for association with CHD in the INTERGENE case-control study.

Subjects with the CC genotype of the -765C>T in the promoter region of IRS2 had an increased risk of developing CHD (n=512 successfully genotyped case-control pairs) (Table II).

To investigate the mechanism behind regulation of IRS2 in foam cell formation, macrophages were exposed to either rosiglitazone, a known agonist of PPARγ, or cyclodextrin, which lowers cellular cholesterol thereby activating SREBP, for 24 h. Rosiglitazone exposure led to increased expression of IRS2 gene expression. Real-time RT-PCR analysis was used to confirm the elevated gene expression of IRS2 in macrophages and foam cells from subjects with atherosclerosis and the observed upregulation in foam cell formation identified in the DNA microarray analysis (Fig. 3A).

Table I. Genes that were differentially expressed in subjects with atherosclerosis compared to control subjects in macrophages and in foam cells.

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Macrophages Ath vs control</th>
<th>Foam cells Ath vs control</th>
<th>Control foam cells/macrophages</th>
<th>Ath foam cells/macrophages</th>
<th>Chromosomal location</th>
<th>Previous QTL linkage studies</th>
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<td>ABCC3c</td>
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<td>0.0456b</td>
<td>0.0000b</td>
<td>17q22</td>
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<td>0.0266b</td>
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<td>0.2200</td>
<td>15q22.3-q23</td>
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<td>0.0301b</td>
<td>0.1021</td>
<td>0.4399</td>
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<td>0.0191b</td>
<td>0.0035b</td>
<td>0.0017b</td>
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<td>0.0248b</td>
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<td>0.0034b</td>
<td>0.0879</td>
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<td>20q11.2-q12</td>
<td>MI (35)</td>
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<td>0.0304b</td>
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<td>0.1041</td>
<td>20q11.2-q12</td>
<td>MI (35)</td>
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<td>FLJ14146</td>
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<td>0.3515</td>
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<td>0.0000b</td>
<td>0.0000b</td>
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<td>0.1789</td>
<td>0.7131</td>
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<td>0.0000b</td>
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<td>KIAA1522</td>
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<td>0.0407b</td>
<td>0.5219</td>
<td>0.0970</td>
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<td>0.0176b</td>
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<td>0.0847</td>
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<td>0.1664</td>
<td>0.0709</td>
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<td>CHD (14)</td>
</tr>
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<td>0.0719</td>
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<td>SLC12A8c</td>
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<td>0.0002b</td>
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<td>0.4998</td>
<td>0.8438</td>
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<td>0.0079b</td>
<td>0.0482b</td>
<td>0.1359</td>
<td>17p13-p12</td>
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</table>

Genes are listed by official gene symbol (OMIM). EPB41L1 is listed twice since two probe sets met the criterion. P-values for the comparisons between groups are listed for each gene; *P-value <0.05. **Gene expression of 7 of the 27 regulated genes was also significantly affected in foam cell formation in both the atherosclerotic and the control group. The chromosomal location of each gene is listed, and previously linkage studies to these chromosomal regions are also included when applicable. Ath, subjects with atherosclerosis; FCHL, familial combined hyperlipidemia; Homol, homology to mice susceptibility loci Athsq2; MI, myocardial infarction; CAD, coronary artery disease; CHD, coronary heart disease.
of IRS2 (Fig. 3B) whereas cyclodextrin had no effect on IRS2 expression (data not shown).

Expression in human carotid plaques. To investigate the in vivo protein expression of IRS2 in atherosclerotic plaques, immunohistochemical staining of human carotid endarterectomies (n=4) with an anti-IRS2 antibody was performed (Fig. 4). IRS2 protein expression (A) co-localized with macrophage-rich sections identified by an antibody against CD68 (B). In addition, IRS2 was also expressed in
endothelial cells facing lumen and endothelial cells in neovascularization (E and F).

Discussion

The aim of this study was to identify novel susceptibility genes for atherosclerosis and CHD using expression profiles of human macrophages. Twenty-seven genes were identified as differentially expressed between macrophages from subjects with atherosclerosis and macrophages from control subjects. Three genes were selected for further study: IRS2, CD86 and SLC11A1.

First, we tried to establish a link between the altered gene expression in macrophages from subjects with atherosclerosis and macrophages from control subjects and genetic variability. We found that subjects with the CC genotype of the -765C>T polymorphism in the IRS2 promoter region had higher gene expression than subjects with the CT or TT genotype in foam cells, with a similar tendency in macrophages. Subjects with the CC genotype had 1.2-fold higher expression than nonCC in foam cells. This is in line with a previous study by Iwamoto et al., who found that the -765C>T substitution decreased IRS2 promoter activity in cell lines using a luciferase assay, where the T allele exhibited 80% expression compared to the C allele (13).

The repeat element in the SLC11A1 promoter region was first described by Searle and Blackwell (14), who identified four different alleles, that have an impact on gene expression (18). Since SLC11A1 gene expression was higher in macrophages from subjects with atherosclerosis than in macrophages from control subjects, we genotyped these subjects for the promoter repeat element. Subjects homozygous for allele 2 had significantly higher gene expression than non22-subjects, however only 3 of 27 successfully genotyped subjects were homozygous for allele 2. This is contrary to the results reported by Searle and Blackwell (14), who reported higher expression of allele 3 using a luciferase assay.

The effect of CD86 on atherogenesis is unclear and poorly investigated. CD80 and CD86 double-deficient mice generated on a LDLR-/- background developed decreased atherosclerosis (10), but LDLR-/- mice receiving bone marrow from CD80-/-CD86-/- mice grew larger atherosclerotic plaques (15). Genotyping of the rs2715267 SNP in the promoter region of CD86 showed no association with gene expression.

We thereafter genotyped the subjects of the INTERGENE population study. Subjects homozygous for the C allele of the -765C>T SNP in the promoter region of IRS2 had a 40% increased risk of CHD. This is a rather modest risk increase, which is in line with the assumption that several genes with rather small individual impact contribute to the total risk for atherosclerosis and CHD. For example, three recent studies associated the 9p21.3 loci with CAD (16-18), with odds ratio estimations varying between 1.3-1.6.

The association between IRS2 and CHD led us to explore this finding further. We assessed the mechanism behind the induction of IRS2 gene expression during foam cell

Figure 4. IRS2 expression in human carotid endarterectomy atherosclerotic plaques. Full section image of staining with antibodies against IRS2 (A) and CD68 (B). The arrows point to the same macrophage-rich area in subsequent sections. A negative control section with omitted primary antibody (C). High magnification image of IRS2 staining in a macrophage-rich area of the plaque (D), endothelial cells lining the vascular lumen (E) and endothelial cells in plaque microvessels (F).
formation. Our finding that rosiglitazone induces IRS2 expression in macrophages suggests that induction of IRS2 in foam cell formation at least in part may be mediated through PPARγ, which is known to be induced by oxidized LDL (19). Previous studies have shown that PPARγ increases IRS2 gene expression in adipocytes (20). In contrast, the addition of cyclodextrin to macrophage culture media did not affect IRS2 gene expression. Cholesterol depletion activates SREBP (21), a known suppressor of IRS2 in the liver (22). However, since we did not observe any decrease in IRS2 gene expression in cyclodextrin-treated macrophages, this suggests that IRS2 is differentially regulated in macrophages compared to the liver.

To investigate IRS2 protein expression in vivo we used immunohistochemistry to verify that IRS2 protein is expressed in macrophages in human carotid plaques, and we also found that IRS2 is expressed in endothelial cells facing lumen and in neovascularization.

IRS2 belongs to the insulin receptor substrate family, a group of proteins that mediate intracellular signalling in response to insulin, insulin-like growth factor 1 and various cytokines (23). The implication of IRS2 in the context of atherosclerosis has been studied only to a limited extent in animal models, and the underlying mechanisms are poorly understood. It has been established that IRS2−/− mice develop diabetes (28,29). The insulin-resistant IRS2−/− mice have enhanced neo-intima formation in response to injury and have significantly higher triglyceride and cholesterol levels, as well as higher systolic blood pressure than wild-type mice (24). IRS2−/−ApoE−/− mice have increased atherosclerosis compared to ApoE−/− mice (25). On the other hand, ApoE−/− mice lethally irradiated and reconstituted with ApoE−/−IRS2−/− haematopoietic cells have smaller lesions than mice reconstituted with ApoE−/−IRS2−/+ cells (11). These findings indicate a difference in systemic and peripheral function of IRS2 in the development of atherosclerosis. This is also in line with studies showing that IRS2 has different functions in different cell types and tissues. For example, Sadagurski et al showed that IRS2 can act both as an activator and a suppressor of glucose transport in a cell-specific manner (26). IRS2 polymorphisms have previously been linked to both obesity (27) and diabetes (28), which in combination with our findings indicates that normal function of this gene is required for a balanced and healthy metabolism.

In conclusion, our screening procedure to identify novel susceptibility genes for atherosclerosis and CHD proved to be successful. IRS2 gene expression was higher in macrophages and foam cells from subjects with atherosclerosis compared to macrophages and foam cells from control subjects, and IRS2 expression co-localised with macrophages in atherosclerotic plaques in vivo. Subjects with the CC genotype of the -765C-T SNP in the promoter region of IRS2 were associated with higher macrophage IRS2 gene expression and an increased risk of CHD. We conclude that IRS2 may be a new susceptibility gene for atherosclerosis.

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