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Genetic mapping of modifier loci affecting malignant hypertension in TGRm*Ren2* rats

Surasak Kantachuvesiri, Chris S. Haley, Stewart Fleming, Katarna Kurian, Caroline E. Whitworth, Philip Wenham, Yuri Kotelevtsev, and John J. Mullins

Centre for Genome Research, University of Edinburgh; Roslin Institute; Department of Pathology, University of Edinburgh Medical School; Department of Renal Medicine, Royal Infirmary of Edinburgh; and Department of Clinical Biochemistry, Western General Hospital, Edinburgh, Scotland, United Kingdom

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Background. Genetic background has a major influence on the manifestation of multifactorial diseases such as hypertension in which severe complications may be caused through an interaction with additional factors, which may be genetically determined. We have previously described a genetic model of malignant hypertension (MH) in rats carrying the mouse *Ren2* gene (TGRm*Ren2*-27), in which the phenotype is dependent on the genetic background.

Methods. Using a single homozygous TGRm*Ren2*-27 male as transgene donor, we produced two F_1 populations with (*a*) 100% penetrance of MH in progeny heterozygous for the Fischer F344 genetic background and (*b*) 58.5% penetrance in progeny heterozygous for the Lewis genetic background. To identify the modifier loci affecting the phenotype, a cohort of 252 males was produced by breeding the same single male with Fischer-Lewis F_1 females. The progeny were phenotyped for clinical and pathological features of MH.

Results. Genome-wide screening and quantitative trait loci (QTL) analysis identified two loci, on chromosome 10 (LOD 4.4) and on chromosome 17 (LOD 3.9) close to the *Ace* and *At1* genes, respectively, which contribute to the lethal MH phenotype. Their influence on mortality was consistent with a multiplicative effect of the two loci. In addition, we found higher plasma angiotensin-converting enzyme activity in progeny receiving the Fischer allele than in progeny receiving the Lewis allele (123.5 $\pm$ 9.5 vs. 91.8 $\pm$ 4.9 U/liter, $P < 0.01$), suggesting the association of angiotensin-converting enzyme and MH.

Conclusions. Our study demonstrates the application of a transgene as a "major gene" to facilitate the identification of modifier loci, which can affect the phenotype of MH, and reveals *Ace* and *At1* as candidate genes involved in the manifestation of the MH phenotype.

Key words: gene mapping, hypertension, cardiovascular, transgenic rat, renin-angiotensin system.

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Malignant hypertension (MH) is a severe form of hypertension with end organ damage occurring in 1 to 2% of the hypertensive population [1–3]. The increasing frequency of diagnosis of mild-to-moderate hypertension and the widespread use of antihypertensive medication have generally resulted in a decreased incidence of MH and an improvement in the prognosis of affected patients [4]; however, in some populations, MH has failed to decline [5].

Clinical markers of transformation to the accelerated phase include a rising blood pressure, a presumed pressure diuresis, renal failure, and the development of grade III or IV retinopathy [3]. The severe increase in blood pressure may cause endothelial damage and proliferation of myointimal cells, resulting in pathological changes in the vasculature, including "onion skinning" with endothelial swelling and fibrinoid necrosis [6, 7]. As a consequence, renal ischemia triggers the activation of the renin-angiotensin system (RAS), causing a further elevation in arterial blood pressure. Moreover, enhanced shear stress in the severely narrowed microcirculation may further endothelial injury and platelet aggregation, which sustain the microangiopathic process.

The predisposing and initiating factors of malignant-phase hypertension are not well understood. The level of hypertension alone may not be the sole factor determining the development of this complication because there is a considerable overlap in actual blood pressures between MH patients and benign hypertensives [8]. The racial difference in the incidence of malignant nephrosclerosis and hypertension-associated end-stage renal failure in black compared with white hypertensive patients even after the adjustment for socioeconomic variables [9, 10] suggests the primary role of genetic predisposition. It is conceivable that one or more genetic polymorphisms in the individual may interact in response to high blood pressure and may determine the severity of vascular injury.

Genetic studies of animal models can identify the candidate genes and provide an insight on the genetic inter-

actions causing phenotypic variation in the human population. We have recently described a rat model of MH that results from the expression of a mouse *Ren2* renin transgene [11]. The penetrance of the MH phenotype was found to be highly dependent on the genetic background, and the pathophysiological changes in MH rats were very similar to those observed in the human disease [11, 12]. To identify modifier genes of MH, we used the transgene to stimulate the phenotype in a diverse genetic background and found the linkage of the MH phenotype to two loci, containing the candidate genes *Ace* and *At1*, which may contribute to the severity of the MH phenotype.

METHODS

Animal crosses and phenotype assessments

Fischer (Fischer F344), Lewis, and Fischer-Lewis F_1 female rats were obtained from Harlan-Olac (Oxford, UK). The transgenic rat TGRm*Ren2*-27 is a hypertensive rat strain that expresses the mouse *Ren2* renin gene and that is maintained on a Sprague-Dawley (SD) background [13]. We minimized the potential for variation in penetrance because of different SD alleles by using a single male homozygous TGRm*Ren2*-27 rat (known to be 90% homozygosity based on our analysis of microsatellite markers) to sire all of the offspring studied. On the basis of our previous data [12], male progeny were chosen for this investigation.

All animals used in this study were bred at the Center for Genome Research (University of Edinburgh, Edinburgh, United Kingdom). Rats were housed with free access to food and water throughout the experimental period and were fed standard commercial rat chow supplied by Special Diet Services (Witham, Essex, UK). Regular 12-hour diurnal cycles were maintained. The breeding, maintenance, and study of animals were performed according to home office regulations. Tail cuff plethysmography was performed under light halothane anesthesia at six and eight weeks of age prior to the development of MH, as previously described [12]. The rats that developed symptoms of MH were killed by either CO_2 inhalation or cervical dislocation. Blood was collected in a heparinized tube and centrifuged at 4°C for six minutes. The plasma was kept at −70°C for determination of angiotensin-converting enzyme (ACE) activity. The kidneys were removed and fixed in 10% formal saline prior to histopathological study using hematoxylin and eosin (H&E), periodic acid-Schiff, and Martius Scarlet Blue (MSB)-stained sections as previously reported [12]. The sections were examined for malignant vascular injury, defined by fibrinoid necrosis and myointimal proliferation of arteries and arterioles, by two pathologists (S.F. and K.K.) without prior knowledge of survival and genetic data.

Genetic markers and fluorescent-dUTP genotyping

Seventy-two animals, 36 survivors (≥120 days of age) and 36 with MH at an early age, were studied using 125 informative markers that covered more than 90% of the rat genome at less than 20 cM intervals. Candidate loci that showed a LOD score of more than 1.5 were then analyzed further by genotyping all 252 animals. The polymorphic markers between Lewis and Fischer strains were chosen with reference to published information (genetic map [14], http://www.genome.wi.mit.edu) and allele size information from Genosys Biotechnologies (Cambridge, UK). Polymerase chain reaction (PCR) primers for the markers were obtained from Research Genetics (Huntsville, AL, USA) and Genosys Biotechnologies (Cambridge, UK). PCR reactions were performed in flexible Thermo-Fast 96-well plates (Advanced Biotechnologies, Surrey, UK) in a total volume of 10 μl containing 150 ng of template DNA, 330 nM of each primer, 200 μM dNTPs, 1 × buffer (Promega, Southampton, UK), 0.5 U of *Taq* polymerase (Promega), and 1.5 mM $MgCl_2$. The ratio of F-dUTP to dTTP was 1:400 for R110 and R6G and 1:200 for TAMRA (Applied Biosystems, Warrington, UK). Reactions were overlaid with 40 μl of light mineral oil and amplified on an Omnigene thermocycler (Hybaid, Middlesex, UK) using the following protocol: initial denaturation at 94°C for five minutes, followed by 30 cycles at 94°C for one minute, 55°C for one minute, 72°C for 1.5 minutes, and final extension at 72°C for ten minutes. The PCR products from five microsatellite markers with nonoverlapping allele size and that were labeled with different fluorescent dyes were pooled and phenol-chloroform extracted. Three and one-half microliters of the loading cocktail, containing 1:5 (vol/vol) of size standard (GENESCAN 500-ROX), formamide/blue dextran and ethylenediaminetetraacetic acid (EDTA) as in the manufacturer's protocol, was added to 1.5 μl of the pooled samples. The samples were then heated at 95°C for three minutes, and 2 μl of the sample were loaded onto an individual gel lane. Electrophoresis was carried out using 4% acrylamide/6 M urea, 36 cm (36 cm Well-to-Read) gels, using a model 377 Sequencer (Applied Biosystems). Analysis was performed using the GeneScan™ 2.0.0 software and Genotyper™ (version 1.1; Applied Biosystems) as described in the manufacturer's manual. Data were exported and stored in Excel (version 4.0).

Measurement of angiotensin-converting enzyme activity

Plasma ACE activity was measured kinetically on a Cobas Mira Plus analyzer using the substrate furylacrylylphenylalanylglycylglycine (FAPGG; Sigma, Dorset, UK) and the method of Maguire and Price [15]. FAPGG is hydrolyzed by ACE to furylacrylylphenylalanine and glycylglycine, which results in a decrease in absorbance

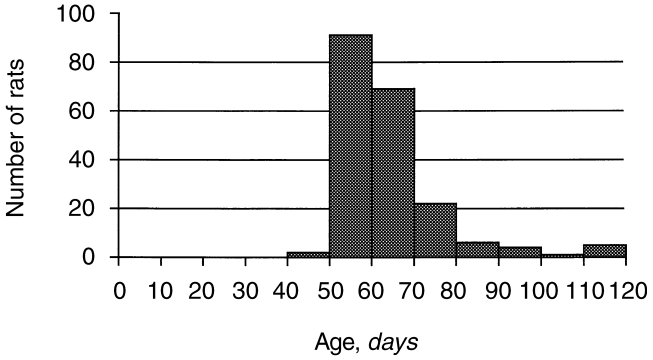

Fig. 1. Distribution of age at time of death of the progeny of TGRm*Ren2*-27 and F_1 (Fischer-Lewis). Out of 252 male progeny, 200 had died by 120 days in total over a range of 49 to 119 days. The median age at time of death was 60 days, with a mean of 63.3 days ± SD 12.2 days.

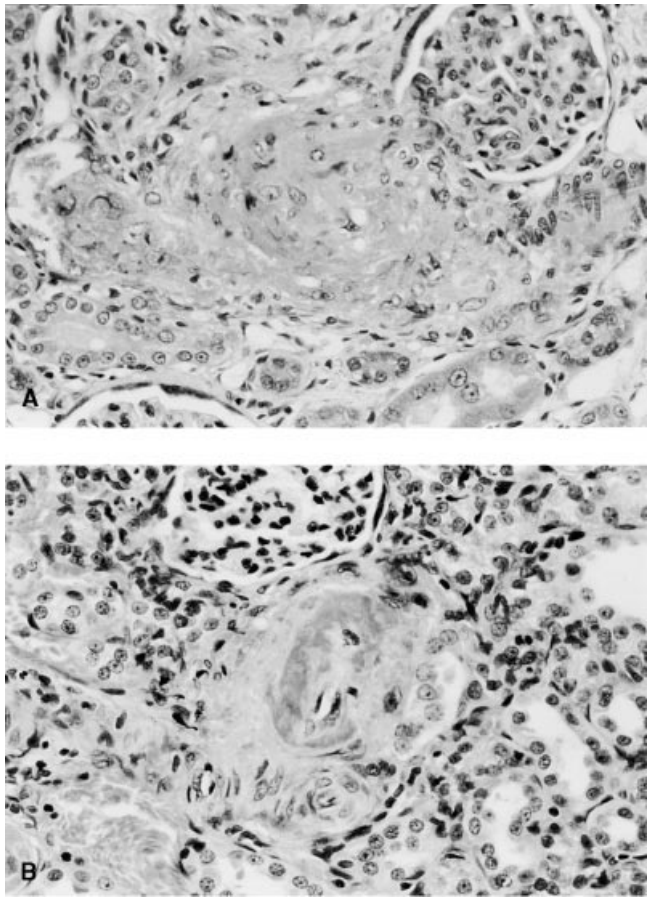

Fig. 2. Histopathology of kidney. The kidneys of animals exhibiting clinical symptoms of malignant hypertension show myointimal proliferation and fibrinoid necrosis of arteries and arterioles. (*A*) Hematoxylin and eosin. (*B*) Martius Scarlet Blue.

at 340 nm, from which the ACE activity can be calculated. The samples were studied in duplicate, and the average of the two measures was used in the analysis.

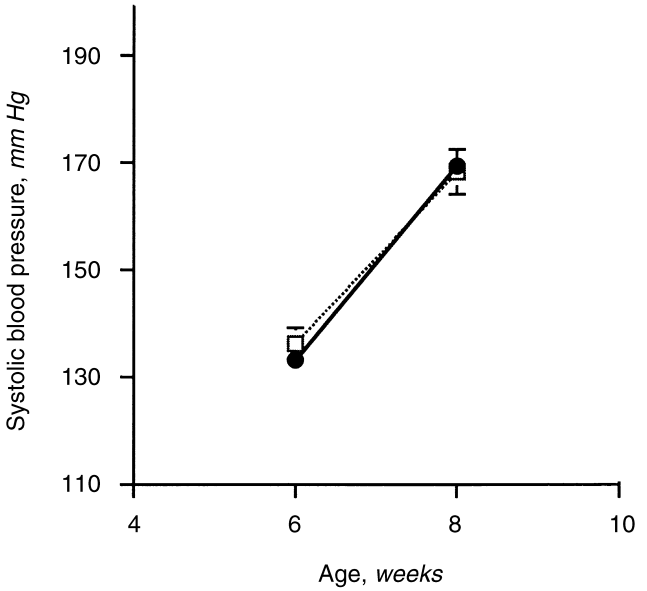

Fig. 3. Tail cuff systolic blood pressure (SBP) measurement of the progeny of TGRm*Ren2*-27 and F_1 (Fischer-Lewis) prior to the development of malignant hypertension (MH). Data are expressed as mean ± SEM. Symbols are: (●) MH; (□) survivors.

Statistical analysis

The genetic map and orders of the markers were obtained for each chromosome using Cri-map [16]. Potential genotyping errors were identified on the basis of inconsistency with Mendelian inheritance and unlikely double recombination events. The genotypes involved were repeated, and where necessary, the data were reanalyzed. The linkage and quantitative trait loci (QTL) analyses were performed by considering phenotypes as "survival" (no clinical symptoms of MH by 120 days of age) or the "age of death (AoD)," respectively. Interval mapping analysis for QTLs was performed using least squares methods, which have been found to be powerful and robust for analysis of both normal and binary data [17, 18]. LOD values from these analyses were calculated from transformation of the F statistic [17]. Dam and parity of litter were included as fixed effects in the analyses. LOD values were compared with a genome-wide significance threshold for a backcross of 3.3 [19]. We used odds ratio (OR) and its confidence interval (CI) to estimate the strength of association [20]. The comparison of mean between groups was made using unpaired *t*-test and two-tailed *P* values. Data are expressed as mean ± SEM.

RESULTS

The malignant hypertensive phenotype in genetic crosses

We bred a single homozygote male transgenic rat TGRm*Ren2*-27 to Fischer and Lewis female rats and

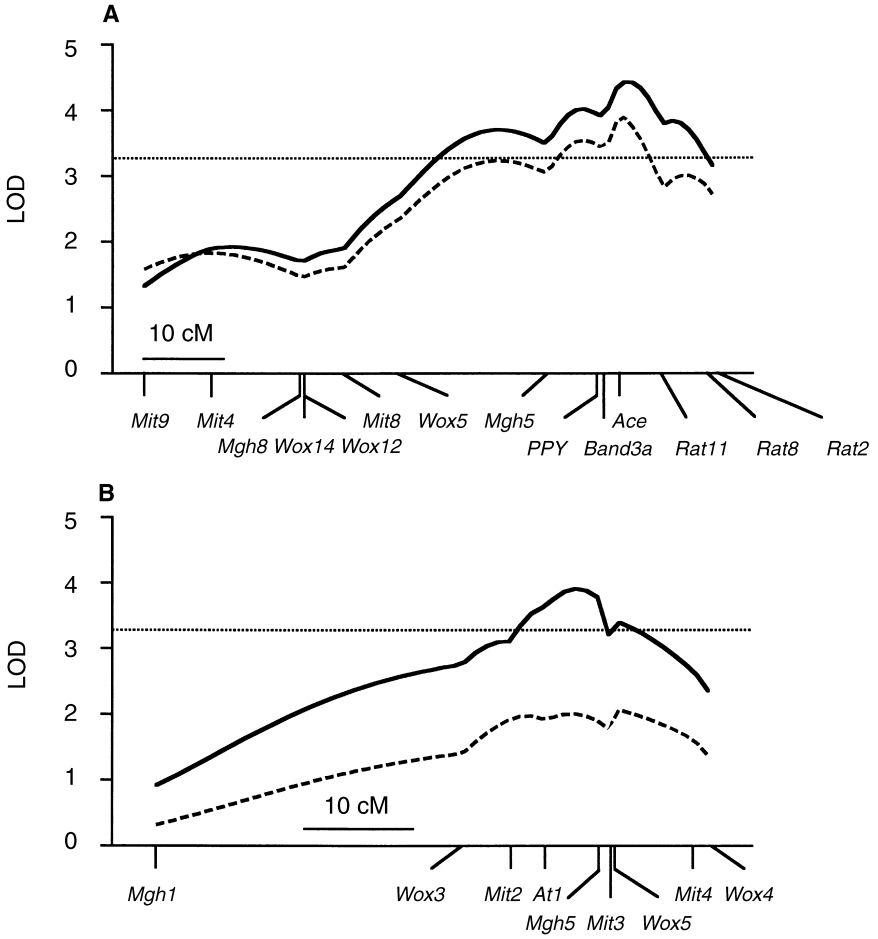

Fig. 4. LOD score of the markers on chromosomes 10 (*A*) and 17 (*B*). The highest LOD scores were found close to the *Ace* and *At1* loci. LOD values for the traits survival (broken line) and age of death (AoD; solid line) are from interval mapping analysis [17, 18]. The broken, horizontal line is the LOD threshold for significant linkage [19] (cM, centiMorgan).

monitored the clinical symptoms of MH, which include polyuria, weight loss, lethargy, piloerection, hemiparesis, seizure, and lethality in the progeny. With a single exception (83 days), all male progeny from Fischer females developed terminal MH between 50 and 70 days of age (mean age 56.9 ± 5.6 days), whereas 41.5% of Lewis progeny survived over 120 days without symptoms (mean AoD in Lewis progeny developing MH was 62.0 ± 6.2 days; Table 1). To genetically map the polymorphic genetic loci conferring susceptibility to MH and mortality, we crossed F_1 Fischer-Lewis hybrid females to the same single homozygous TGRm*Ren2*-27 SD male. All progeny were obligate *Ren2* heterozygotes and, for any given locus, carried one SD allele and a second allele of either Fischer or Lewis origin. Among the 252 male progeny obtained and phenotyped, 79.4% developed MH (mean age 63.3 ± 12.2 days; Fig. 1). Those showing no symptoms by 120 days were scored as "survivors."

Histological examination showed the presence of fibrinoid necrosis, intimal proliferation, and acute nephron injury in all animals (Fig. 2). Additional features in those who had survived beyond 120 days were nephrosclerosis, tubular atrophy, and fibrosis of arterial walls. Blood pressure was determined at six and eight weeks of age prior to the development of MH and showed no significant difference in systolic blood pressure between the survivors and the MH group (Fig. 3).

Linkage and quantitative trait loci analyses

We screened 125 informative microsatellite markers distributed over the entire rat genome. QTL analyses of either "survival," as a binomial trait, or "AoD," as a quantitative phenotype, identified linkage with two chromosomal regions. The first locus was identified on chromosome 10 at or close to the *Ace* gene with a LOD score of 3.9 for "survival" and 4.4 for "AoD" (Fig. 4). This locus reduced the life span by 7.3 ± 1.6 days and increased the probability of MH by 4.2-fold ($P < 0.0001$ by univariate analysis) in animals inheriting a Fischer allele in comparison to those inheriting a Lewis allele.

The second locus was identified on chromosome 17 in the region close to the gene encoding the type 1 angiotensin receptor, *At1*, at which the LOD score for "AoD" was 3.9 (Fig. 4). The LOD score for "survival" was maximal in the same chromosomal region but did not reach significance (LOD 2.1). At this locus, the inheritance of a Lewis

Table 1. Incidence of lethal malignant hypertensive phenotype (MH) in three heterozygote crosses produced by breeding a single male TGRm*Ren2*-27 to females of different strains

Heterozygote crosses	MH (*N*)	Survivors (*N*)	% MH
TGRm*Ren2*-27 × F_1 (Fischer-Lewis)	200	52	79.4
TGRm*Ren2*-27 × Fischer	53	0	100
TGRm*Ren2*-27 × Lewis	31	22	58.5

Table 2. Survival analysis with reference to the genotype of *Ace* (chromosome 10) and *At1* (chromosome 17) loci

Genotype		MH	Survivors	
Ace	*At1*	(*N*)		Odds ratio (95% CI)
LS	FS[a]	40	28	1
LS	LS	48	12	2.8 (1.3–6.2)
FS	FS	57	9	4.4 (1.9–10.4)
FS	LS	55	3	12.8 (3.6–45.2)
Total		200	52	

FS represents the Fischer/Sprague-Dawley haplotype and LS represents the Lewis/Sprague-Dawley haplotype.
[a] Reference group

allele rather than a Fischer allele reduced the life span by 6.9 ± 1.6 days and increased the probability of MH by 2.6-fold ($P = 0.006$ by univariate analysis). The genome-wide screening found no significant evidence for linkage of either "survival" or "AoD" to other chromosomes (all LOD scores were less than 1.5). Moreover, there was no effect of both loci on the trait "blood pressure" at six and eight weeks, as detected by QTL analysis.

Epistasis of linkage loci

To test for interaction between the *Ace* and *At1* loci, whose products act downstream of renin in the RAS, we analyzed "survival" with reference to the genotype of the *Ace* and *At1* loci. The risk of lethality from MH increased 2.8-, 4.4-, and 12.8-fold in animals carrying susceptibility alleles on chromosomes 17, 10, and both chromosomes, respectively, when compared with animals without the susceptibility loci (Table 2), and these data are consistent with a multiplicative effect of the two loci.

Plasma angiotensin-converting enzyme activity in different F_1 progeny

To test the hypothesis of the association of the *Ace* gene and its product in the development of MH phenotype, we measured plasma ACE activity in Fischer and Lewis progeny. We found a higher plasma ACE activity in F_1 progeny of TGRm*Ren2*-27 × Fischer than in F_1 progeny of TGRm*Ren2*-27 × Lewis (123.5 ± 9.5 vs. 91.8 ± 4.9 U/liter, $P < 0.01$, $N = 15$ and 16).

DISCUSSION

We applied genetic analysis to map modifier loci influencing the MH phenotype caused by the interaction between the *Ren2* transgene and the Fischer F344 and Lewis genetic backgrounds. We found linkage of MH with loci on chromosomes 10 and 17 close to the *Ace* and *At1* genes, respectively. Both *Ace* and *At1* can be considered as candidate genes contributing to lethality from MH, because the RAS is known to be involved in the pathophysiology, and subhypotensive doses of ACE inhibitors prevent mortality from MH in this model [21]. The *Ace* locus has previously been shown to determine plasma ACE activity but had no direct effect on blood pressure in an F_2 intercross between the stroke-prone spontaneously hypertensive rats ($SHRSP_{HD}$) and the normotensive Wistar-Kyoto (WKY_{HD}-0) reference strain [22]. It is possible that altered expression of the *Ace* gene in Fischer rats contributed to development of MH and mortality, and we observed higher plasma ACE activity in TGRm*Ren2*-27 × Fischer F_1 progeny than in TGRm*Ren2*-27 × Lewis F_1 progeny. Furthermore, the association of the deletion polymorphism of the *Ace* gene and end organ damage in human cardiovascular and renal disease has been well documented [23–29]. ACE may regulate vasoreactivity and vascular response to hypertensive injury by altering the concentration of angiotensin II, which is known to have effects on cell growth and extracellular matrix formation [30–32] and thereby affect the processes leading to organ damage and mortality from MH.

Quantitative trait loci analysis suggested that a locus on chromosome 17 at or near the *At1* gene, which encodes the angiotensin II receptor type I, may affect the timing of mortality from MH. The finding that down-regulation of this receptor occurs in TGRm*Ren2*-27 rats [33] supports the hypothesis that the *At1* gene may play a role in the MH phenotype, possibly by genotype-specific differences in receptor regulation. The interaction between the effects of the loci on chromosome 10 and 17 further supports the hypothesis that both the *Ace* and *At1* genes are likely to be candidate genes determining the lethal MH phenotype, and indeed the interaction between these loci has been found in human myocardial infarction [34]. From our previous studies [11, 12], we found a low penetrance of MH in the "Hannover" SD background, and it was clear that "Hannover" SD alleles did not contribute to the MH phenotype in a dominant manner. Those data are consistent with our study in which the data suggest that the Fischer locus on chromosome 10 and the Lewis locus on chromosome 17 have a dominant effect on the MH phenotype.

Quantitative trait loci analysis showed no linkage of loci on chromosomes 10 and 17 with blood pressure at six and eight weeks of age. Moreover, we found no difference in blood pressure between survivors and the MH group prior to the development of MH, which was consistent with the previous report [12]. These data suggest that

blood pressure prior to the transition to malignant-phase hypertension is not a predicting factor for the development of MH, and that additional factors may play a role in the initiation and/or severity of the phenotype.

We cannot exclude the possibility that in our cross, the variation in penetrance of MH was due to the inheritance of different parental SD alleles. However, because the single male TGRm*Ren2*-27 used to establish the cohort proved to be homozygous for all markers localized within the 95% CI of the maximum LOD scores on chromosomes 10 and 17, this possibility is unlikely. Because of the constraints placed on the design of this study, the presence of recessive loci could not be directly tested.

By using the transgene to induce hypertension in different strains of rats, we identified a polygenic inheritance of MH lethality involving two modifier loci from the Fischer and Lewis genetic backgrounds. These loci contain candidate genes encoding ACE and the angiotensin II receptor type 1. The generation of appropriate congenic strains may provide a useful resource for more refined genetic analysis of the MH phenotype and the identification of the genes involved. In addition, it might be of interest to study the polymorphism of these candidate genes in human MH, which may help to identify patients at risk of MH and may potentially allow modification of this risk by pharmacological intervention.

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Reprint requests to John J. Mullins, Ph.D., Department of Medical and Radiological Sciences, Wilkie Building, University of Edinburgh Medical School, Teviot Place, Edinburgh EH8 9AG, United Kingdom. E-mail: J.Mullins@ed.ac.uk

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