ELECTRONIC LETTER

Single nucleotide polymorphism (SNP) analysis of mouse pulmonary adenoma susceptibility loci 1–4 for identification of candidate genes

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Lung cancer is the leading cause of mortality from cancer in both men and women in developed countries. There is evidence that, although incidence is almost always associated with environmental factors such as smoking or occupational exposure, susceptibility has a genetic component with early onset lung cancer following Mendelian inheritance.^{1,2} Moreover, susceptibility is largely intrinsic to the lung itself as shown by the classical experiments involving lung explants from sensitive and resistant mice.³ Identification of the genes predisposing to cancer could yield targets for treatment or chemoprevention. In humans, the wide variety of carcinogens and varying degrees of exposure make identifying the predisposing genes difficult, but in a mouse model, such confounding variables can be controlled.

Previously, classical genetic studies involving cross breeding of mouse strains with differing susceptibilities have identified

chromosomal areas associated with predisposition to developing spontaneous and chemically induced lung adenomas.^{4,5} The process involves use of many evenly spaced polymorphic DNA markers to create landmarks across each chromosome. In the process called quantitative trait loci (QTL) mapping, attempts are made to find significant correlations between marker alleles and the phenotypic variation, or disease state.⁶ Inbred mouse strains vary in their susceptibility to cancer and two extreme strains are A/J (susceptible) and C57BL/6J (B6, resistant).^{4,5} Four QTLs identified as pulmonary adenoma susceptibility (Pas) loci 1–4 have been mapped, respectively, to mouse chromosomes 6, 17, 19, and 9. These regions are illustrated in fig 1.

Much genetic variation between people is a result of random mutation at specific nucleotide positions. These single nucleotide polymorphisms (SNPs, pronounced snips) have the potential to produce profound effects on gene expression and consequently phenotype, as shown in fig 2. For example, SNPs in the 3' UTR region can alter the stability of the mRNA, by changing binding sites or secondary structure, thus making it more or less likely to be degraded. A SNP in the 5' region can change promoter binding sites and thereby modify the affinity for a transcription factor. Nonsense mutations are the most severe of the SNPs found in the coding region. Nonsense SNPs are ones that introduce a premature stop codon resulting in a truncated polypeptide, often resulting in loss of function. Missense mutations result in an amino acid change that can be important if the properties of the new amino acid (charge, polarity, etc) are different from the one it replaced. As susceptibility to lung cancer has a strong genetic component, detailed SNP analysis of polymorphisms between A/J and B6 (A-B SNPs) may facilitate the identification of candidate susceptibility genes. The genomes of both strains have been sequenced and assembled, and thus direct comparison is now possible.

Celera's mouse reference SNP database (1.0), the first such resource to become available, includes 2 566 706 SNPs and is based on the first *Mus musculus* assembly release which includes the publicly produced sequence of C57BL/6J and the Celera produced sequences of A/J, DBA/2J (DBA), 129X1/SvJ, and 129S1/SvImJ. The database was scanned for A-B SNPs in the Pas1–4 QTLs. The resulting list of SNPs was analysed to identify the most likely candidate genes. This SNP analysis complements previous gene expression analysis and, as in that work, builds on classic genetic results and integrates newly available genomic data.⁷

Key points

- Past studies have mapped four susceptibility loci (Pas1–4) for pulmonary adenoma in which A/J and C57BL/6J (B6) mice have different alleles that affect incidence and multiplicity of tumours. With the release of a genome wide SNPs database, it has become feasible to analyse these genetically determined QTLs for genes polymorphic in these strains.
- Celera's discovery database (CDS 3.6, SNP 1.0) was scanned for SNPs in the Pas1–4 QTLs. SNPs were first screened according to the following criteria: (1) A/J and B6 were polymorphic for the SNP; (2) SNPs appeared in the coding region, the 5' regulatory region, or the 3' untranslated region; (3) SNPs appeared in known genes; (4) B6 and DBA/2J, phenotypically similar to B6, shared the same allele.
- Genes for which associations or other plausible links with cancer have been published were deemed as final candidates. All 11 selected SNPs within candidate genes were verified by polymerase chain reaction (PCR) sequencing. We have also attempted to verify a series of differentially expressed candidate susceptibility genes to lung tumours in our previous microarray analysis with semiquantitative reverse transcriptase PCR (RT-PCR).
- Differential expression of six of seven candidate genes were confirmed. Candidate Pas genes are Pas1, *receptor type protein tyrosine phosphatase* and *basic helix-loop-helix B3*; Pas2, *Notch4*, *CREBL1*; Pas3, *Minpp1* and *FoxD4*; and Pas4, *TGF β receptor II*. Identification of the genes predisposing to mouse lung cancer could have considerable implications for diagnosis, treatment, or chemoprevention of lung cancer in humans.

Abbreviations: Pas, pulmonary adenoma susceptibility; PCR, polymerase chain reaction; QTL, quantitative trait loci; RT-PCR, reverse transcriptase PCR; SNPs, single nucleotide polymorphisms; Scc1, susceptibility to colon cancer 1

Figure 1 Mouse pulmonary adenoma susceptibility loci (Pas) as mapped in crosses of strains A/J and C57BL/6. Diagrams show chromosomes drawn to a scale of physical length according to Celera CDS 3.6. Pas1 appears at the distal end of chromosome 6 with genetic position of the flanking markers at the left and the marker names and key genes at the right. Pas2–4, respectively on chromosomes 17, 19, and 9, are similarly labelled.

MATERIALS AND METHODS

Approach

Markers flanking each QTL were located in the Celera (Rockville, MD) mouse genome database (www.celera.com, CDS 3.6 release). These markers were used to identify the reference DNA positions within the assembled genome for our database query. The Celera mouse SNP reference database (v1.0) was queried for SNPs within each QTL then filtered to keep only A-B SNPs.

Data filtering

Additional filtering of the A-B SNPs was performed to increase confidence in positive results. Often with screening one seeks to reduce the false negative rate and thereby be inclusive, but owing to the tremendous number of SNPs (~20 000 in Pas1 alone), we sought to minimise the false positive rate. To accomplish this, A-B SNPs that met the following criteria were selected for further analysis: (1) it appeared in the coding region, the 5' regulatory region,

3' UTR SNP

nnnnTGGGAGACC**G**CAnnn
 Palindromic:
 2° hairpin structure
 could stabilise mRNA

nnnnTGGGAGACC**CC**Annn
 Lost palindrome,
 less stable mRNA

5' UTR SNP

nnnn(T/G)(T/A)CGTCAnnnnn
 Cyclic AMP
 response element
 binding site

nnnn(T/G)(T/A)CTTCAnnnnn
 Binding site lost

Missense SNP

–Thr–**Cys**–Leu–His–
 nnnnACTTGCTGCACnnn

–Thr–**Tyr**–Leu–His–
 nnnnACTTGCTGCACnnn

Nonsense SNP

–Thr–**Cys**–Leu–His–
 nnnnACTTGCTGCACnnn

–Thr–**STOP**
 nnnnACTTGATTGCACnnn

Figure 2 Hypothetical examples of biologically effective single nucleotide polymorphisms (SNPs). Disease producing SNPs in the untranslated regions may influence mRNA stability in the case of 3' UTR (top panel) or promoter activity in the 5' UTR (second panel). Among the coding SNPs, missense SNPs (third panel) could cause an amino acid change that, for example, removes a disulphide bridge. Nonsense SNPs, by definition, introduce truncations that can diminish or obliterate protein function.

or the 3' untranslated region; (2) it appeared in a known gene; (3) the DBA (another resistant strain) allele was the same as the B6 allele. Intronic, intergenic, and silent SNPs were excluded. This was done for two reasons: (1) the function of intronic and intergenic sequence is a matter of research beyond the scope of this study; (2) the number of these SNPs made their analysis currently untenable. For the resulting genes containing A-B SNPs, further analysis was performed to sequence verify the SNPs with PCR sequencing analysis, and to identify those genes or gene families in which association with lung cancer has been previously established.

Table 1 Summary of SNPs found in Celera Mouse SNP Reference database (v 1.0). Total refers to the number of all SNPs (any type, any strain-strain polymorphism) in the region. Filtered refers to the number of SNPs meeting the criteria described in the text. "No of genes" refers to the number of genes associated with the filtered SNPs. Known genes are any genes having specific functional annotation in the Celera CDS 3.6 database

Locus	Total	Filtered	No of genes	Known Genes	No of genes
Pas1	19 092	99	65	16	15
Pas2	19 639	69	37	25	14
Pas3	26 916	96	55	16	11
Pas4	21 505	147	88	24	14

Table 2 A-B SNPs in known Pas1 genes. SNPs appear in order of position on the chromosome. SNP ID is Celera's unique ID number for the SNP. GenBank refers to the transcript sequence associated with the gene containing the SNP. Gene name is derived from Celera's annotation wherever possible. Type refers to the gene structure containing the SNP and, for coding SNPs, it refers to the effect on amino acid sequence predicted by the most authoritative reading frame(s) for the transcript. Chr refers to the base pair position of the SNP on the chromosome according to the Celera CDS 3.6 assembly. mRNA refers to its nucleotide position within the corresponding Celera transcript. Allele/codon describes the nucleotide change, and, when it occurs within the translated region, it is described in terms of the codon triplet. C57BL/6J alleles always appear on the left, A/J alleles on the right. DBA refers to concordance of DBA/2J allele with B6 allele. RT is checked when the SNP was confirmed by RT-PCR sequencing. Array refers to whether the gene was found differentially regulated in a previous microarray study [Lemon WJ *et al.* *J Med Genet* 2002;39:644-55]. Pb is checked when published reports have associated the gene with cancer. Description is derived from either Celera annotation or GenBank annotation, whichever the authors deemed more clear, with deference to Celera.

SNP ID	GenBank	Gene name	Type	Relative position						DBA	SNP RT	Pb	Description
				Chr	mRNA	Codon	AA	Allele/codon	AA				
mCV22281390	BM489316	<i>Arpc4</i>	Missense	112,205,003	48	GGC GTC	Gly Val	G/C	Gly Val				Actin related protein 2/3 complex, subunit 4
mCV22584830	NM_009525	<i>Wnt5b</i>	3' UTR	118,266,570	1158	A/G						Y	Wingless related MMV integration site 5B
mCV25250691	NM_008646	<i>Mug2</i>	NonSense	120,923,657	4417	TGA CGA	Stop Arg		Stop Arg	Y			Murinoglobulin 2
mCV25250751	M65237	<i>Mug1</i>	5' UTR, missense	120,988,713	788/808	GTA GCA	Ala Val						Murinoglobulin (MUG3)
mCV24835024	NM_009645	<i>Aicda</i>	3' UTR	121,357,737	1703	G/A							Activation induced cytidine deaminase
mCV24136983	NM_027218	<i>1810046124Rik</i>	Missense	121,870,184	618	AAA AAC	Asn Lys			Y			C type lectin
mCV22899606	NM_010736	<i>Lbr</i>	3' UTR	123,978,076	1445	G/A							Lymphotoxin B receptor
mCV25230505	NM_007376	<i>A2m</i>	Missense	127,183,453	1670	ATT GTT	Ile Val		Ile Val	Y			Alpha2-macroglobulin
mCV22524446	NM_031499	<i>Pip2</i>	Missense	130,318,725	5252	CAA GAA	Glu Gln			Y			Proline rich protein 2
mCV24262005	NM_008462	<i>Klra2</i>	3' UTR	133,237,819	1237	C/T					Y		Killer cell lectin-like receptor, subfamily A, member 2
mCV23359715	NM_026371	<i>5830457120Rik</i>	3' UTR	134,662,880	1095	T/A							
mCV23359716	NM_025589	<i>2410038A03Rik</i>	3' UTR	134,663,400	1615	A/G				Y			
mCV24045161	NM_011216	<i>Pipr</i>	Missense	136,486,200	201	GAT GGT	Asp Gly		Asp Gly	Y			Protein tyrosine phosphatase, receptor type, O
mCV23381606	NM_024469	<i>Bhlhb3</i>	3' UTR	137,526,924	1929	-/T				Y			basic helix-loop-helix domain containing, class B3
mCV24099286			Missense	145,935,561	812	TCG CCG	Ser Pro		Ser Pro	Y			

Table 3 A-B SNPs in Pas2 known genes. Columns as described for table 2

SNP ID	GenBank	Gene name	Type	Relative position			DBA	SNP RT	Description
				Chr	mRNA	Allele/codon			
mCV24100604	NM_020581	Angpl4	Ins/del	33,532,739	1318	G/-			Angiopoietin-like 4
mCV24103348	NM_030697	O610013D04Rik	Missense	33,577,259	1809	TCT CCT	Ser Pro	Y	
mCV24105091	NM_016761	Klf1	3' UTR	33,635,219	1545	G/C		Y	Kinesin family member C1
mCV24107583	NM_020603	Bing4	Missense	33,693,845	118	ACC GCC	Thr Ala	Y	
mCV24297946	NM_010929	Notch4	Missense	34,676,126	3014	GAA GAC	Glu Asp	Y	Sequence of the mouse major histocompatibility locus class II region
mCV24296263	NM_017406	Creb1	Missense	34,745,777	662	AAC AGC	Asn Ser	Y	Common integration site for the mouse mammary tumour virus
mCV24296262			Missense	34,745,896	697	TCT GCT	Ser Ala	Y	cAMP responsive element binding protein-like 1, ubiquitous transcription factor
mCV24295985	NM_031176	Trnb	Missense	34,767,693	1838	CAC CGC	Asn Arg	Y	Tenascin XB
mCV22965429	NM_020027	Bat2	Missense	35,246,204	6470	CAC CGC	His Arg		HLA-B associated transcript 2 (aka HLA-G2)
mCV22965384			Acceptor splice site	35,246,540	6243	T/C		Y	
mCV23273805	NM_008199	H2-Bf	NonSense	36,632,166	1023	TAG CAG	Asn Gln		Histocompatibility 2, blastocyst - expressed at the blastocyst stage & placenta
mCV23273810			Missense	36,634,076	827	AAG AAT	Lys Asn		
mCV23273811			Missense	36,634,087	816	GAG CAG	Asn Gln		
mCV23273815			Missense	36,634,119	784	GCC GGC	Gly Ala		
mCV23273817			Missense	36,635,104	180	GGT AGT	Gly Ser		
mCV24433115	NM_025520	2310034K10Rik	3' UTR	36,698,819	387	T/C		Y	
mCV24433812	NM_024468	Rnf23	3' UTR	36,732,547	1929	C/T			Tripartite motif protein 39
mCV24436558	NM_019439	Gabrr1	Missense	37,495,966	2476	ACA GCA	Thr Ala	Y	Gamma-aminobutyric acid (GABA-B) receptor, 1, note: putative CpG island
mCV23715424	BM490573	2610528M18Rik	Missense	41,344,966	396	GTA ATA	Val Ile	Y	
mCV23715425			Missense	41,344,981	381	GTT ATT	Val Ile	Y	
mCV25200870	NM_032003	Enpp5	Missense	44,507,091	1530	CAT AAT	His Asn		Ectonucleotide pyrophosphatase/phosphodiesterase 5
mCV25200871			3' UTR	44,507,302	1741	G/A		Y	
mCV25200881			3' UTR	44,507,604	2043	A/G			
mCV25200882			3' UTR	44,507,769	2208	C/-			

* A/J allele not reported in Celera SNP Reference Database 1.0.

Table 4 A-B SNPs in Pas3 known genes. Columns as described for table 2

SNP ID	GenBank ID	Gene name	Type	Relative position		Allele/codon	AA	DBA	SNP RT	Pb	Description
				Chr	mRNA						
mCV23294255	NM_007650	<i>Cd5</i>	3' UTR	8,418,081	1811	T/C		Y			CD5 antigen
mCV23294266			3' UTR	8,418,184	1708	T/C		Y			
mCV23294598			Missense	8,426,359	100	GTC ATC	Val Ile				
mCV23010981	NM_025658	<i>2210417J23Rik</i>	Missense	9,221,967	437	GTT GCT	Val Ala				Guanine nucleotide binding protein, alpha q polypeptide Annexin A1
mCV24416503	NM_008139	<i>Gnaq</i>	3' UTR	14,037,739	1241	T/G					
mCV23044840	NM_010730	<i>Anxa1</i>	Missense	18,100,327	946	GAA GGA	Glu Gly				
mCV24486668	NM_008022	<i>Foxd4</i>	Missense	22,674,812	1698	CAG CGG	Gln Arg		Y		Forkhead box D4
mCV24486689			Missense	22,675,284	1226	GGG CGG	Gly Arg	Y	Y	Y	
mCV22823123	NM_028785	<i>1200017A24Rik</i>	3' UTR	22,988,476	2509	A/G					Very low density lipoprotein receptor Cleavage stimulation factor, 3' pre-RNA subunit 2
mCV23196871	NM_013703	<i>Vldlr</i>	Missense	25,022,920	1118	GGG AGG	Gly Arg				
mCV23093281	NM_031249	<i>Cstf2t-pending</i>	3' UTR	28,869,655	2056	G/C		Y			
mCV23094584	NM_018830	<i>Asah2</i>	5' UTR	29,849,129	306	T/G		Y			N-acylsphingosine amidohydrolase 2
mCV25400117	BB002878	<i>Minpp1</i>	Nonsense	30,268,848	564	TGA TGG	Stop Trp		Y		Multiple inositol polyphosphate histidine phosphatase 1
mCV25441217	NM_026487	<i>4921525H23Rik</i>	3' UTR	30,455,931	1840	C/T		Y			
mCV25441216			3' UTR	30,455,946	1825	G/A		Y			
mCV25441200			3' UTR	30,456,513	1258	A/G					

Table 5 A-B SNPs in Pas4 known genes. Columns are as described for table 2

SNP ID	GenBank	Gene Name	Type	Relative position		Allele/codon	AA	DBA	SNP RT	Pb	Description
				Chr	mRNA						
mCV24278514	NM_013669	<i>Snap91</i>	UTR 5	80,912,581	855	G/A					Synaptosomal associated protein, 91
mCV24278524			UTR 5	80,912,656	780	A/G					
mCV23421071	NM_025360	<i>1200002G13Rik</i>	UTR 3	86,317,238	768	T/C					Procollagen lysine, 2-oxoglutarate 5-dioxygenase 2 Kyphoscoliosis
mCV25420569	NM_011961	<i>Plod2</i>	UTR 3	89,191,569	2,231	C/T					
mCV23564270	NM_024291	<i>Ky</i>	UTR 3	99,085,849	4114	A/G					
mCV23564271			UTR 3	99,085,879	4144	G/A					Aminoacylase 1 Steroid receptor-interacting SNF2 domain protein
mCV23586226	NM_025371	<i>1110014J22Rik</i>	Missense	103,062,990	533	GTT GCT	Val Ala	Y			
mCV23592429	NM_030730	<i>Srsnf2t-pending</i>	UTR 3	103,313,283	4819	G/A		Y	Y		
mCV23592440			Missense	103,314,355	3747	GTC ATC	Val Ile		Y		Hyaluronidase 2 Interferon related developmental regulator 2
mCV24367303	NM_010489	<i>Hyal2</i>	UTR 3	104,181,371	1730	T/C		Y		Y	
mCV22576892	NM_025903	<i>lfrd2</i>	Missense	104,196,642	152	TCC CCC	Ser Pro				
mCV22576749			Missense	104,198,775	329	GCC ACC	Ala Thr				TRAF interacting protein Cell division cycle 25 homologue A Chemokine (C-C) receptor 1-like 2
mCV22576540	BM486632	<i>Sema3b</i>	UTR 3	104,207,874	2139	T/A			Y		
mCV25006051	NM_011634	<i>Traip</i>	Missense	104,559,428	46	GCC ACC	Ala Thr				
mCV25024853	NM_007658	<i>Cdc25a+1154</i>	Missense	106,607,499	384	CAC CAA	His Gln		Y		Transforming growth factor, beta receptor II
mCV23789456	NM_017466	<i>Cmkbr1l2</i>	UTR 5	107,780,575	190	C/A					
mCV23789449			UTR 5	107,780,576	189	C/T					
mCV23789448			UTR 5	107,780,703	62	G/A					Transforming growth factor, beta receptor II
mCV23789447			UTR 5	107,780,748	17	A/C					
mCV23303603	NM_009371	<i>Tgfb2</i>	UTR 3	113,551,165	2312	T/G		Y*	N*	Y	
mCV23303602			UTR 3	113,551,334	2143	C/T		Y*	N*		
mCV22520381	NM_025974	<i>3100001N19Rik</i>	Missense	118,043,888	568	GTT GCT	Val Ala				
mCV22520379			UTR 3	118,044,104	784	A/G					
mCV22520373			UTR 3	118,044,148	828	T/C					

*See table 3.

SNP annotation

The SNP IDs were used to link SNPs with the genes in which they occur and Celera's mechanism to link its genes with public sequences was used for reporting public accession numbers and descriptions from GenBank (RefSeq when possible).

Sequence verification of SNPs

A-B SNPs were sequence verified with RT-PCR sequencing by the sequencing core facility in the Division of Human Cancer Genetics, Ohio State University. Briefly, the transcript sequence for the candidate genes were downloaded from the Celera database and PCR primers flanking the SNPs were designed such that 200–400 bp fragments were produced. RT-PCR, as described later, was performed to amplify total RNA harvested from lungs of A/J and C57BL/6J mice. These amplified fragments were submitted to the sequencing core facility where they were processed according to standard procedures.

Confirmation of differential gene expression by RT-PCR

Expression of several candidate genes found here by SNP analysis, or previously by array analysis,⁷ were verified by RT-PCR. RNA was harvested from normal lungs of A/J, BALB/cJ, and C57BL/6J mice, reverse transcribed and amplified by PCR. A mixture of 4 µg of total RNA, oligo (dT) primer (Invitrogen, Grand Island, NY, USA), and double distilled water was incubated at 70°C for 10 minutes. This mixture was then added to the reverse transcription reaction mixture containing the following: forward reaction buffer, DTT (Invitrogen, Grand Island, NY, USA), RNasin (Promega, Madison, WI, USA), dNTPs, and M-MLV reverse transcriptase (Invitrogen, Grand Island, NY, USA). This final mixture was incubated at 37°C for one hour. Next, a final incubation at 95°C for two minutes was performed to remove RNA.

For real time PCR, a LightCycler-DNA Master SYBR Green I kit (Roche, Indianapolis, IN, USA) and an iCycler iQ (Biorad, Rodeo, CA, USA) thermocycler were used. Each reaction on a 96 well microtitre plate contained 2 µl of cDNA template and 2 µl of master mix. The kit master mix consisted of *Taq* DNA polymerase, reaction buffer, dNTP mix, SYBR Green I dye, and 10 mmol/l MgCl₂. The final reaction had a 1 µmol/l concentration of forward and reverse primers and 2 mmol/l MgCl₂ concentration, and a final volume of 20 µl. The PCR variables were as follows: a heating temperature of 95°C, an annealing temperature of 55°C, and an elongation temperature of 72°C were used for 34 cycles.

The genes examined were *SmarcD3*, *ATFa*, *Cdk5*, *PDCD4*, *Cdc25A*, and *Mbd2* together with β actin as a control. Base 2 logarithm of fold changes were computed, for gene x between strains A and B, as $\log_2(x_A) - \log_2(\beta \text{ actin}_A) + \log_2(\beta \text{ actin}_B) - \log_2(x_B)$.

RESULTS

Our approach to identifying candidate susceptibility genes for lung tumours with SNPs complements our earlier work with gene expression analysis.⁷ As in the previous study, this work builds on existing genetic mapping of QTLs, availability of the mouse genome sequence, and SNP data. The genome sequence, combined with previously identified QTLs, allows focus on SNP analysis within the regions known to modulate susceptibility and resistance.

Fig 1 illustrates the physical positions of the Pas QTLs, highlights the flanking markers and shows the corresponding genetic positions. As expected, proximal regions span less genetic distance than equal length distal regions, owing to the higher distal recombination rate. Table 1 shows the number of SNPs found in each QTL and the number of genes containing SNPs in each QTL for each step in the filtering process. For example, 19 092 SNPs were found in Pas1. After removing the silent, intronic, and intergenic SNPs and those not A-B polymorphic, the number was reduced to 99 A-B SNPs associ-

ated with 65 genes. After the final step of isolating only the A-B SNPs in known genes, 16 SNPs in 15 genes remained.

Tables 2–5 enumerate, for each Pas QTL, the A-B SNPs found in known genes. Of the 15 A-B genes containing SNPs in Pas1, two stand out as strong candidates: *Ptpro* and *Bhlhb3*. In both genes, the B6 allele is shared with DBA and the sequence of the SNP for *Ptpro* has been verified. *Ptpro* is a receptor type O protein, tyrosine phosphatase. It has been suggested that other members of this family are tumour suppressor genes, including the recent suggestion that *Ptprj* (mouse chromosome 2) is the susceptibility gene to colon cancer 1 (*Sccl*).⁸ *Bhlhb3* contains a basic helix-loop-helix domain and is expressed in late embryogenesis and may be a target of *Notch* ligands.^{9,10}

Pas2

In this locus, often noted for the preponderance of HLA genes, two non-HLA genes stand out. *Notch4*, as a known oncogene,¹¹ and *CrebL1*, as a member of the cyclic AMP response element binding proteins, could contribute to metastatic potential of melanoma cells.^{12,13} The A/J allele for the *Notch4* SNP was not reported in the Celera database, but sequencing showed it to vary between A/J and B6. Both seem to have a functional relation to tumorigenesis and the B6 allele is shared with DBA.

Pas3

Two genes were found to be candidates, *Minpp1* and *FoxD4*. The sequences of SNPs for both genes were verified. *Forkhead box D4* is the best candidate, having one B6 allele shared with DBA and being a member of a family of transcription factors that regulate the cell cycle, and it may play a part in tumorigenesis.¹⁴

Pas4

TGFβRII has historically been the primary candidate for Pas4. Both B6 alleles are shared with DBA, but the A/J allele is not reported in the Celera database. As an SNP was found in Celera between B6 and 129X1, we checked the region for a SNP between A/J and B6, but found none. It has been shown that loss of the type II receptor is often associated with a loss of TGF-β induced cell cycle repression.¹⁵ Reduction of type II receptor mRNA is strongly associated with lung adenocarcinoma in mice and humans.^{15,16} However, RT-PCR sequencing showed no SNP between A/J and B6.

Figure 3 Comparison of microarray data with RT-PCR for selected genes. Fold change of gene expression between A/J and C57BL/6J for five genes and between A/J and BALB/cJ for one gene (*Mbd2*) are compared on a log₂ basis. Each unit on the y axis represents a twofold change. Negative values represent higher expression in B6.

RT-PCR verification of microarray data

Fig 3 shows RT-PCR follow up on selected genes found to be candidates in our previous microarray study.⁷ In that study, RNAs from normal mouse tissue from these strains were evaluated for differential expression across strains. Genes within the QTLs that are differentially expressed across strains were deemed as candidates on the basis that expression differences could predispose to tumorigenesis. However, the microarray data were not confirmed by conventional RT-PCR or northern blotting techniques. In this study, we selected seven of the differentially expressed candidate genes found in that study for further confirmation. Six of the seven genes examined by RT-PCR showed differential expression similar to that found with the array, as shown in fig 3. One gene, *ATFa*, did not show the same pattern with RT-PCR and array. Note that this gene had the lowest level of expression of the seven genes (Li-Wong full model estimates of gene expression: B6=27, A/J=58). Fig 3 shows the correspondence of differential expression measured in the array data with that found by RT-PCR and correspondence between the two assay systems is consistent for six of seven candidate genes assayed.

DISCUSSION

Following the commonly held and straightforward view that genetic determinants of susceptibility may arise from the simplest form of genetic variation, the SNP, we analysed SNPs in four susceptibility loci to pulmonary adenoma. Five strains have been sequenced and assembled to date: A/J (susceptible, s), DBA/2J (resistant, r), C57BL/6J (r), and two substrains of 129, namely 129X1/SvImJ (intermediate, i) and 129S1/SvJ (i).^{5,17} Substrain 129X1/SvJ was contaminated in 1978 and, as a result, contains as much as 25% non-129 DNA. Owing to consequent large scale heterozygosity, it is considered by some as not an inbred strain.^{18–20} Manenti *et al*¹⁷ reported 129X1/SvJ to be of intermediate susceptibility which leaves the expectation for Pas genotypes ambiguous. Similarly strain 129S1, the “steel” strain, is an inbred strain, but being of intermediate susceptibility, the expectation for Pas genotypes is also ambiguous. Consequently, our criteria required that DBA and B6 share alleles and that A/J be different, but the 129 alleles were ignored. Analysis was limited to SNPs likely to be detectable in mRNA, namely, coding SNPs, splice site SNPs, and UTR SNPs. As *K-ras*, which has been a strong Pas1 candidate gene for some time, has polymorphisms between A/J and B6 in intron 2,⁵ we would have liked to include intronic SNPs. However, so many intronic SNPs were identified that it was deemed at this point untenable to sort out the useful ones.

Pas1 has been shown to be the major locus, accounting for 45% of the phenotypic variance.⁴ Mutant *K-ras* is found in 80% or more of mouse lung adenomas and adenocarcinomas with mutations of codons 12 and 61 reported in both susceptible and resistant strains.^{21,22} In hybrid animals, mutation occurs at a much higher frequency in susceptible alleles than in resistant alleles.²³ A bioassay in the same study showed that introduction of the mutant allele, derived from either the susceptible or resistant parent, was sufficient to transform NIH 3T3 cells. Recent studies have confirmed the strong association of the locus with cancer in mice and have shown that the homologous human locus, 12p12, is a major susceptibility locus in Italian and Japanese people.^{17,24–26} The entire region between *K-ras* and *ITPR2* is homologous between mouse chromosome 6 and human chromosome 12, and human *K-ras* has been shown to share mutations associated with cancer at codons 12 and 61.²⁷ Some questions have remained. By what mechanism is the A/J allele of *K-ras* susceptible? Is there another gene in Pas1 that confers the propensity to mutation? Perhaps it is the *Par2* gene on chromosome 18, which has been shown to modulate the effect of Pas1? According to the present SNP analysis of Pas1, the *protein tyrosine phosphatase* and the *basic helix-loop-helix B3* genes are also candidates. Additional functional analysis of

the allelic differences in cell transformation and carcinogenesis should provide more definitive answers.

Pas2 QTL is located at the H-2 locus the haplotypes of which correlate with the incidence and multiplicity of induction of mouse lung tumour. The primary candidates for this region are *Notch4* and *Creb1*. *Notch4* has been shown to be susceptible to viral activation, which can transform cells and drive them to a highly invasive phenotype.¹¹ This gene was also a candidate in previous expression studies. *Creb1*, a SNPs binding protein, has been implicated in transcriptional activation and may contribute to the acquisition of metastatic phenotypes.¹³

Pas 3 was first described by Devereux *et al*²⁸ as flanked by D19MIT42 and D19MIT19. They used linkage analysis of N-ethyl-N-nitrosourea treated (A/J × C57BL/6J) F1 × C57BL/6J backcross progeny. This finding was later confirmed by Festing *et al*²⁹ with urethane treated (A/J × C57BL/6J) F2 mice. The main candidate in the Pas3 region is *Foxd4*. *Foxd4* is a forkhead transcription factor. Forkhead transcription factors are involved in regulating cell cycle progression and cell death. Long term Forkhead activation causes a sustained but reversible inhibition of proliferation without a marked increase in apoptosis.¹⁴

Finally, Pas4 was mapped by Festing *et al*.²⁹ One of the candidates is *TGFβRII*, the expression of which is reduced in urethane induced pulmonary adenoma in A/J mice. Loss of type 2 receptor results in a loss of induction of apoptosis.³⁰ Loss of sensitivity to *TGFβ* is a hallmark of invasiveness in some cancers.³¹ However, no SNP between A/J and B6 was found, suggesting that the basis for its candidacy as Pas4 may be intronic or intergenic.

In this study, we also used RT-PCR to corroborate the results for six of seven candidate genes identified by allelic specific differential expression in our earlier study (fig 3).⁷ Other work by us with others showed similar corroboration between RT-PCR and oligonucleotide arrays.^{32,33} This strengthens the case for these genes potentially to play a part in predisposition of lung tumorigenesis in mice. However, the differences between these assays in reported fold change suggests that array results should continue to be taken cautiously and RT-PCR validation of the most important results should continue. Replication of arrays on multiple animals is also recommended. The study on gene expression complements the present study on SNPs in two ways. Firstly, differences in gene expression may not be the result of SNPs, so the gene expression based candidates bring in new information. Secondly, SNPs can have full effect without any change in gene expression whatsoever. So the sets of candidates are not mutually exclusive, nor are they subsets. Instead they are complementary.

In summary, we have described several candidates for the Pas and Par QTLs using the SNP analysis and microarrays. These candidates should accelerate identification of susceptibility genes to mouse lung tumour and potential functional interactions among different Pas and Par QTLs. This and our previous study show the synergies between classical genetic studies and genomic tools such as the SNP database and microarrays in dissecting the genetic basis of susceptibility to lung tumour in mice.

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