Identification of Susceptibility Loci in a Mouse Model of KRAS\textsuperscript{G12D} -Driven Pancreatic Cancer

Tonia C. Jorgenson\textsuperscript{1}, Bret R. Williams\textsuperscript{1}, Allyson Wendland\textsuperscript{2}, Andrea Bilger\textsuperscript{1}, Eric P. Sandgren\textsuperscript{2}, and Norman R. Drinkwater\textsuperscript{1}

Abstract

Genetic background affects susceptibility to pancreatic ductal adenocarcinoma in the Ela-KRAS\textsuperscript{G12D} mouse model. In this model, KRAS oncogene expression is driven by an elastase promoter in acinar cells of the pancreas on an FVB/NTac (FVB) background [FVB-Tg(Ela-KRAS\textsuperscript{G12D})] with the transgene carried on the \textit{Y} chromosome. Through linkage analysis of crosses between the C57BL/6J (B6), BALB/c (BALB), and DBA/2J (D2) inbred strains of mice and resistant FVB-Tg(Ela-KRAS\textsuperscript{G12D}), we have identified six susceptibility loci that affect mean preinvasive lesion multiplicity. Markers on chromosome 2 segregated with high tumor multiplicity in all three strains; these loci were designated Prsq1-3 (pancreatic ras susceptibility quantitative trait loci 1-3; combined F2 and N2 LOD\textsubscript{WS} 6.0, 4.1, and 2.7, respectively). Susceptibility loci on chromosome 4, designated Prsq4 and Prsq5, were identified in crosses between FVB transgenic mice and B6 or BALB mice (combined F2 and N2 LOD\textsubscript{WS} 3.6 and 2.9, respectively). A marker on chromosome 12 segregated with tumor multiplicity in a BALB × FVB-Tg(Ela-KRAS\textsuperscript{G12D}) cross and was designated Prsq6 (LOD\textsubscript{WS} ∼2.5). B6-Chr \textit{Y}\textsuperscript{FVB-Tg(Ela-KRAS\textsuperscript{G12D})} and BALB-Chr \textit{Y}\textsuperscript{FVB-Tg(Ela-KRAS\textsuperscript{G12D})} consomics, which carry the KRAS transgene on the FVB \textit{Y} chromosome on an otherwise inbred B6 or BALB background, developed ∼4-fold (B6) and ∼10-fold (BALB) more lesions than FVB-Tg(Ela-KRAS\textsuperscript{G12D}) mice. By 12 months of age, 10% of BALB-Chr \textit{Y}\textsuperscript{FVB-Tg(Ela-KRAS\textsuperscript{G12D})} mice developed invasive carcinomas. Our findings provide evidence that regions of chromosomes 2, 4, and 12 influence the development and progression of pancreatic neoplasms initiated by an oncogenic allele of KRAS in mice.

Development of PDAC is influenced by a combination of genetic events (somatic mutations) and nongenetic events (tissue damage) in humans as well as in animal models (3, 4). Activating mutations of the KRAS oncogene, which can be found in >90% of PDAC (5), as well as inactivating mutations of the tumor suppressor genes CDKN2A, TP53, and SMAD4, have been identified (3). These alterations accumulate over time and are associated with progression of the disease through consecutive stages of pancreatic intraepithelial neoplasia (PanIN; refs. 6–8). In the late stages of PDAC, identification of the potential roles (if any) of the many mutations that have accumulated becomes very difficult. The creation of mouse models to investigate the multistep progression model has greatly expanded our knowledge (9, 10). The diverse genetically engineered models, which use a variety of promoters to target multiple exocrine cell types, cause a spectrum of pathologic changes that mimic various aspects of the human disease (11). These models have provided evidence that activated KRAS might be an initiating genetic event, and that it cooperates with CDKN2A and TP53 deficiency to cause progression to metastatic disease (9–12).

KRAS is a member of the \textit{RAS} family of oncoproteins involved in the signaling pathways of many receptor tyrosine kinases. The mutations most commonly seen in PDAC, substitution of either valine or aspartate in place of glycine at codon 12, mimic the GTP-bound form or active conformation of KRAS (13–15). A transgenic mouse model directing oncogenic
KRAS activity to the pancreas, *Ela-KRAS*^{G12D}, has been generated on an FVB inbred genetic background and described previously (16). The elastase promoter sequence directs expression of human *KRAS*^{G12D} specifically to the acinar cells of the exocrine pancreas. Animals harboring this transgene have normal pancreases at birth, but by 1 month, multiple focal preneoplastic lesions are observed. Adult mice display acinar-to-ductal metaplasia and preinvasive ductal lesions.

We crossed *Ela-KRAS*^{G12D} mice with several divergent inbred strains to identify genetic modifiers that affect the multiplicity and progression of pancreatic lesions. We report the discovery, through linkage analysis, of the *pancreatic ras susceptibility quantitative trait loci* 1-6 (*Prsq1-6*). These loci regulate the risk for formation and progression of pancreatic ductal neoplasms.

Materials and Methods

**Mice**

C3H/HeJ (C3), SWR/J (SW), C57BL/6J (B6), DBA/2J (D2), and BALB/cJ (BALB) inbred strains were obtained from The Jackson Laboratory. FVB/NJ mice were obtained from Taconic. FVB-*Tg(Ela-KRAS)*^{G12D} transgenic mice [FVB-*TgN* (Ela1*Kras*{G12D})9EPS], which carry the transgene on the Y chromosome, were described previously (16). All mice were used in accordance with the NIH Guide for the Care and Use of Laboratory Animals. All experimental protocols were approved by the Animal Care and Use Committee of the University of Wisconsin-Madison. Mice were housed in plastic cages on corncob bedding (Bed O'Cobs, Anderson Cob Division). Mice used in the initial F1 study (Supplementary Table S1) and cages on corncob bedding (Bed O'Cobs, Anderson Cob Division). Mice used in the initial F1 study (Supplementary Table S1) and those bred and maintained for subsequent linkage and consomic mice are fed *Mice Diet* 9 F5020 (Lab Diet). All mice were given acidified tap water *ad libitum*. Animal health was monitored by daily inspection.

**Phenotype analysis**

To determine the influence of the genetic background of *Ela-KRAS*^{G12D} mice on pancreatic lesion multiplicity, FVB-*Tg(Ela-KRAS)*^{G12D} male transgenic mice were crossed with C3, SW, B6, D2, and BALB inbred strain females to produce F1 progeny. These strains were chosen to provide a genetically diverse pool for the identification of potential modifier genes (17). Pancreatic lesion multiplicity of F1 progeny was determined at 14 months of age. To map modifier loci in *Ela-KRAS*^{G12D} mice, F1 progeny from crosses between FVB transgenic and B6, D2, or BALB mice were mated to create intercross (F2) mice (FVB × B6, FVB × D2, and FVB × BALB). The F1 progeny were also backcrossed to the FVB parental strain to create backcross (N2) progeny. F2 and N2 progeny were analyzed for pancreatic lesion multiplicity at 12 months of age. Consomic mice carrying the FVB-*Tg(Ela-KRAS)*^{G12D} or FVB-B6-*Tg(Ela-KRAS)*^{G12D} or FVB-BALB-*Tg(Ela-KRAS)*^{G12D} Y chromosome were created by backcrossing to B6 (N17) and BALB (N11) inbred backgrounds, and pancreatic lesion multiplicity was determined at 12 months of age.

Mice were euthanized by CO₂ asphyxiation. Pancreases were collected and fixed in Carnoy's solution for 1 hour and then transferred to 70% ethanol. After fixation, pancreases were bisected and paraffin embedded, sectioned to the first "full face" of pancreatic tissue, mounted on a slide, and stained with H&E for microscopic examination. Slides were then examined using an Olympus BX40 microscope at ×100 magnification, and ducts containing irregular epithelial cells (16) were enumerated. Slide section areas were analyzed with ImageJ v1.42 software (18).

**Genotyping**

DNA was isolated from tails (modifier study and linkage analysis) or toes (consomics) using a standard protease

### Table 1. Pancreatic lesion multiplicity of *Ela-KRAS*^{G12D} mice at 12 mo of age is increased in crosses between FVB and B6, D2, or BALB

<table>
<thead>
<tr>
<th>Mouse strain</th>
<th>No. mice</th>
<th>Lesion multiplicity ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>FVB-<em>Tg(Ela-KRAS)</em>^{G12D}</td>
<td>25</td>
<td>1.6 ± 1.5</td>
</tr>
<tr>
<td>B6FVB-<em>Tg(Ela-KRAS)</em>^{G12D}F1</td>
<td>35</td>
<td>14.9 ± 6.6*</td>
</tr>
<tr>
<td>D2FVB-<em>Tg(Ela-KRAS)</em>^{G12D}F1</td>
<td>34</td>
<td>14.1 ± 5.8*</td>
</tr>
<tr>
<td>BALBFVB-<em>Tg(Ela-KRAS)</em>^{G12D}F1</td>
<td>30</td>
<td>11 ± 15*</td>
</tr>
<tr>
<td>B6FVB-<em>Tg(Ela-KRAS)</em>^{G12D}F2</td>
<td>76</td>
<td>12.1 ± 9.9*</td>
</tr>
<tr>
<td>D2FVB-<em>Tg(Ela-KRAS)</em>^{G12D}F2</td>
<td>74</td>
<td>18 ± 19*</td>
</tr>
<tr>
<td>BALBFVB-<em>Tg(Ela-KRAS)</em>^{G12D}F2</td>
<td>78</td>
<td>15 ± 13*</td>
</tr>
<tr>
<td>FVB(B6FVB-<em>Tg(Ela-KRAS)</em>^{G12D})N2</td>
<td>52</td>
<td>8.9 ± 9.1†</td>
</tr>
<tr>
<td>FVB(D2FVB-<em>Tg(Ela-KRAS)</em>^{G12D})N2</td>
<td>54</td>
<td>7.8 ± 5.6†</td>
</tr>
<tr>
<td>FVB(BALBFVB-<em>Tg(Ela-KRAS)</em>^{G12D})N2</td>
<td>51</td>
<td>6.4 ± 5.1†</td>
</tr>
</tbody>
</table>

*Significantly different from the FVB-*Tg(Ela-KRAS)*^{G12D} value by Wilcoxon rank sum test, *P < 10⁻⁵.*
†Significantly different from the FVB-*Tg(Ela-KRAS)*^{G12D} value by Wilcoxon rank sum test, *P < 10⁻⁶.*
K–ammonium acetate precipitation protocol (19). Microsatellite markers (20) were amplified using 1 μL DNA (∼100 ng) using standard conditions (19). The products were separated by electrophoresis through acrylamide gels. B6FVB-Tg(Ela-KRASG12D)F2 and FVB(B6FVB-Tg(Ela-KRASG12D)F1) N2, D2FVB-Tg(Ela-KRASG12D)F2 and FVB(D2FVB-Tg(Ela-KRASG12D)F1) N2, and BALBFVB-Tg(Ela-KRASG12D)F2 and FVB(BALBFVB-Tg(Ela-KRASG12D)F1) N2 progeny were genotyped at ∼80 markers spanning the mouse genome (Supplementary Table S2).

**Linkage analysis**

We used a nonparametric approach to assess linkage between the marker loci and the quantitative trait loci (QTL) that determine lesion multiplicity (21). The data for each marker were analyzed for F2 and N2 mice using the Jonckheere-Terpstra and Wilcoxon rank sum tests, respectively, to obtain the test statistic $Z_W$ (22). Genome-wide $P$ values were estimated by permutation of the phenotype data (100,000 permutations for each cross) using Qlink 3.2 (19). For each permutation, the most extreme test statistic ($Z_W$) was obtained and this distribution was used to determine the genome-wide significance level (23). Minor QTL were identified by a conditional permutation test, in which lesion data at each marker were stratified according to the genotype at the major QTL. This secondary QTL analysis should also identify interactions between the major and minor loci. Equivalent LOD (logarithm of odds) scores, LOD$_W$ (21), were estimated as LOD$_W = 0.5 \log_{10}(Z_W)$. The significance level for the overall hypothesis of linkage was determined using the method described by Fisher to combine data from F2 intercrosses and N2 backcrosses (24). One-LOD support

<p>| Table 2. Linkage to pancreatic lesion multiplicity in F2 intercross and N2 backcross mice |
|-----------------------------------------------|-----------------------------------------------|------------------|-----------------|</p>
<table>
<thead>
<tr>
<th><strong>Cross</strong>: DNA marker</th>
<th>Chromosome Position (Mb)</th>
<th>LOD$_W$</th>
<th>$P^*$</th>
<th>Lesion multiplicity ± SD (n)†</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>B6FVB-Tg(Ela-KRASG12D)F2</strong>:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D2Mit62</td>
<td>2</td>
<td>118</td>
<td>3.28</td>
<td>0.0056</td>
</tr>
<tr>
<td>D4Mit17</td>
<td>4</td>
<td>63</td>
<td>3.17</td>
<td>0.0013</td>
</tr>
<tr>
<td><strong>FVB(B6FVB-Tg(Ela-KRASG12D)) N2</strong>:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D2Mit62</td>
<td>2</td>
<td>118</td>
<td>2.75</td>
<td>0.016</td>
</tr>
<tr>
<td>D4Mit17</td>
<td>4</td>
<td>63</td>
<td>0.47</td>
<td>NS</td>
</tr>
<tr>
<td><strong>BALBFVB-Tg(Ela-KRASG12D)F2</strong>:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D2Mit51</td>
<td>2</td>
<td>163</td>
<td>1.43</td>
<td>NS</td>
</tr>
<tr>
<td>D4Mit17</td>
<td>4</td>
<td>63</td>
<td>2.84</td>
<td>0.016</td>
</tr>
<tr>
<td>D12Mit5</td>
<td>12</td>
<td>82</td>
<td>0.04</td>
<td>NS</td>
</tr>
<tr>
<td><strong>FVB(BALBFVB-Tg(Ela-KRASG12D)) N2</strong>:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D2Mit51</td>
<td>2</td>
<td>163</td>
<td>2.63</td>
<td>0.023</td>
</tr>
<tr>
<td>D4Mit17</td>
<td>4</td>
<td>63</td>
<td>0.02</td>
<td>NS</td>
</tr>
<tr>
<td>D12Mit5</td>
<td>12</td>
<td>82</td>
<td>2.39</td>
<td>0.046</td>
</tr>
<tr>
<td><strong>D2FVB-Tg(Ela-KRASG12D)F2</strong>:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D2Mit148</td>
<td>2</td>
<td>179</td>
<td>2.49</td>
<td>0.039</td>
</tr>
<tr>
<td><strong>FVB(D2FVB-Tg(Ela-KRASG12D)) N2</strong>:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D2Mit148</td>
<td>2</td>
<td>179</td>
<td>0.16</td>
<td>NS</td>
</tr>
</tbody>
</table>

Abbreviation: NS, not significant.

*Genome-wide significance level.

†Mean number of ductal lesions ± SD (number of mice).

‡FVB/FVB vs FVB/B6: $P < 0.03$; FVB/B6 vs B6/B6: $P < 0.02$.

§FVB/FVB vs FVB/B6: $P < 0.02$; FVB/B6 vs B6/B6: $P < 0.03$.

∥FVB/FVB vs FVB/BALB: $P > 0.12$; FVB/BALB vs BALB/BALB: $P < 0.006$.

¶FVB/FVB vs FVB/D2: $P > 0.8$; FVB/D2 vs D2/D2: $P < 0.0004$.
intervals for the loci identified in these analyses were estimated using R/qtl (25).

**RNA extraction and reverse transcription-PCR**

Two separate methods were used. The first method used pancreatic tissue from F1 and consomic mice that was homogenized with Trizol (Invitrogen) according to the manufacturer’s directions. RNA was visualized by ethidium bromide staining in 1% agarose gels. The absorbance ratio ($260$ nm/$280$ nm) for RNA was between $1.8$ and $2.0$. Reverse transcription was performed using the SuperScript II system (Invitrogen). Primers for the quantitative PCR of $\beta$-actin (control) and the $KRAS/Kras$ cDNAs were designed with Primer3 software (Whitehead Institute for Biomedical Research, Cambridge, MA; Supplementary Table S3) based on the $KRAS$ and $\beta$-actin mRNA sequences (26). Real-time quantitative PCR was performed on total cDNA using the SYBR Green Dye kit (Applied Biosystems). Relative expression values were calculated as the amount of the $KRAS$ mRNA relative to the geometric mean of three housekeeping genes including $Sdha$, $Ywhaz$, and $Actg1$ (28).

**Protein analysis**

Protein was extracted from frozen pancreatic tissue using the TriPure Reagent (Roche). Protein ($50 \mu g$) was loaded and separated on Tris-tricine 10% to 20% 17-well PAGE gels (Fisher) and electrotransferred to nitrocellulose membranes (Osmonics). The membranes were incubated overnight with primary antibodies to detect $\beta$-actin with goat anti-$\beta$-actin (Santa Cruz Biotechnology), Ras with rabbit anti-Ras (Cell Signaling), mitogen-activated protein kinase (MAPK) with antibody p44/42 (Cell Signaling), and phospho-MAPK with antibody p44/42 (Cell Signaling). Membranes were then incubated with horseradish peroxidase–linked goat antibody (Cell Signaling) or rabbit anti-IgG (Cell Signaling) secondary antibodies diluted at 1:1,000. Bands were detected using LumiGLO (Cell Signaling) for detection on film, and the ECL Western Blotting Detection kit (GE Healthcare) for analysis. Protein levels were quantified with the Phosphorimager and calculated as the amount of phospho-MAPK relative to $\beta$-actin.

**Results**

**Genetic background affects pancreatic lesion multiplicity**

To determine whether genetic background affects lesion multiplicity in $Tg(Ela-KRASG12D)$ transgenics, we analyzed lesion multiplicity at 14 months in the male progeny of $FVBYTg(Ela-KRASG12D)$ males mated with C3, SW, B6, D2, or BALB females. Ductal lesions were counted in H&E-stained sections as described (16). Except for the SW cross, the F1 progeny all showed an increase in the formation of ductal lesions relative to $FVBYTg(Ela-KRASG12D)$ mice (Supplementary Table S1). Significant increases relative to $FVBYTg(Ela-KRASG12D)$ mice (0.3 ± 0.6) were identified in B6FVB-Tg(Ela-KRASG12D)$F1$ (11 ± 5; 37-fold effect), D2FVB-Tg(Ela-KRASG12D)$F1$ (8 ± 4; 27-fold effect), BALBFVB-Tg(Ela-KRASG12D)$F1$ (8 ± 4; 27-fold effect), and C3FVB-Tg(Ela-KRASG12D)$F1$ (4 ± 4; 13-fold effect) mice. SWFVB-Tg(Ela-KRASG12D)$F1$ (0.6 ± 1) mice were similar to FVB transgenic mice in susceptibility.

**Modifier loci map to chromosomes 2, 4, and 12**

Crosses between B6, BALB, or D2 mice and FVB transgenic mice were used to map QTls through linkage analysis of intercross (F2) and backcross (N2) mice sacrificed at 12 months. These mice were housed and analyzed separately from those in the preliminary F1 screen described above. The F1 mice in the linkage study developed more lesions but revealed the same relative susceptibilities of the inbred strains. The mean pancreatic lesion multiplicity of control in 12-month-old FVB-Tg(Ela-KRASG12D) mice was $1.6 \pm 1.5$ (Table 1). The increase in lesion multiplicity in F1 mice at 12 months ranged from 7-fold in BALBFVB-Tg(Ela-KRASG12D)$F1$ mice to 9-fold in B6FVB-Tg(Ela-KRASG12D)$F1$ and D2FVB-Tg(Ela-KRASG12D)$F1$ mice (Table 1). F1 mice were intercrossed or backcrossed to FVB. Intercross mice developed 7- to 11-fold

---

**Table 3.** B6-Chr $Y^{FVBYTg(Ela-KRASG12D)}$ and BALB-Chr $Y^{FVBYTg(Ela-KRASG12D)}$ consomic mice develop a significantly larger number of lesions than FVB-Tg(Ela-KRASG12D)

<table>
<thead>
<tr>
<th>Mouse strain</th>
<th>No. mice</th>
<th>Lesion multiplicity ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>FVB-Tg(Ela-KRASG12D)</td>
<td>32</td>
<td>2.8 ± 1.6</td>
</tr>
<tr>
<td>B6-Chr $Y^{FVBYTg(Ela-KRASG12D)}$</td>
<td>32</td>
<td>11 ± 6.4*</td>
</tr>
<tr>
<td>BALB-Chr $Y^{FVBYTg(Ela-KRASG12D)}$</td>
<td>30</td>
<td>28 ± 13*</td>
</tr>
</tbody>
</table>

*Significantly different from the FVB-Tg(Ela-KRASG12D) value by Wilcoxon rank sum test (B6: $P < 10^{-8}$, BALB: $P < 10^{-10}$).
more lesions, on average, than control FVB-\textit{Tg(Ela-KRAS}\textsuperscript{G12D}) mice (Table 1). Backcross progeny exhibited intermediate (4- to 6-fold) increases in the multiplicity of lesions relative to FVB-\textit{Tg(Ela-KRAS}\textsuperscript{G12D}) (Table 1).

To map major QTLs, intercross and backcross genotype data at markers spanning the mouse genome and lesion phenotype data were assessed for linkage using a nonparametric permutation test. Markers on chromosomes 2, 4, and 12 segregated with lesion multiplicity and were designated \textit{Prsq1-3, Prsq4-5, and Prsq6}, respectively. The combined LOD\textsubscript{w} scores for the backcross and intercross mice are shown in Fig. 1.

![Figure 1. Identification of Prsq1-6 by linkage analysis. Genomic DNA samples from the B6 × FVB-\textit{Tg(Ela-KRAS}\textsuperscript{G12D}), D2 × FVB-\textit{Tg(Ela-KRAS}\textsuperscript{G12D}), and BALB × FVB-\textit{Tg(Ela-KRAS}\textsuperscript{G12D}) crosses were genotyped at ~80 microsatellite markers ~20 CM apart spanning the genome. The LOD\textsubscript{w} scores for the F2 and N2 crosses of each strain were combined. Markers on chromosomes 2, 4, and 12 segregated with tumor multiplicity and were designated \textit{Prsq1-3, Prsq4-5}, and \textit{Prsq6}, respectively. The gray horizontal lines indicate the designated genome-wide significance levels.](image)

The three \textit{Prsq} loci on chromosome 2 map to the distal third of the chromosome. \textit{Prsq1} was identified in B6 × FVB crosses and is most significantly linked to D2Mit62 at 118 Mb (\(P < 0.006, \text{F2}; P < 0.016, \text{N2}; \text{Table 2}\)). The B6 \textit{Prsq1} allele is a semidominant susceptibility allele relative to FVB (2.7-fold effect). \textit{Prsq2} was linked to D2Mit51 at 163 Mb in the BALB × FVB backcross (\(P < 0.023, \text{N2}\)). In the BALB × FVB intercross, the same marker was linked to a similar, but not significant, effect. The BALB allele of \textit{Prsq2} confers susceptibility relative to FVB (2.3-fold effect). \textit{Prsq3} was mapped to D2Mit148 at 179 Mb in the D2 × FVB intercross (\(P < 0.039, \text{F2}\)). The D2 \textit{Prsq3} allele causes susceptibility relative to FVB (3.3-fold effect) and it is recessive, consistent with its lack of effect in the backcross. The one-LOD support interval for \textit{Prsq2} (BALB, 142–164 Mb) is contained entirely within that for \textit{Prsq1} (B6, 114–174 Mb; Supplementary Fig. S1). The interval for the \textit{Prsq3} (D2, 162–179 Mb) locus overlaps the distal end of the \textit{Prsq1-2} support interval. Because of the limited overlap of its one-LOD support interval and the smaller effect of the \textit{Prsq3} locus, further characterization of the chromosome 2 modifiers focused on those carried by B6 and BALB.

\textit{Prsq4} is linked to chromosome 4 near 63 Mb in the B6 × FVB intercross (\(P < 0.0002, \text{F2}; \text{Table 2}\)). A similar, but not significant, trend in lesion multiplicity was observed in the B6 × FVB backcross. The B6 \textit{Prsq4} allele is a semidominant resistance allele relative to FVB (2.9-fold effect). \textit{Prsq5} is linked to the same marker in the BALB × FVB intercross (\(P < 0.016, \text{F2}\)). The BALB allele causes susceptibility relative to FVB (2.8-fold effect) and it is recessive, consistent with its lack of effect in the backcross. The one-LOD support interval for \textit{Prsq4} (B6, 58–81 Mb) is contained within that for \textit{Prsq5} (BALB, 54–125 Mb; Supplementary Fig. S1). Lesion multiplicity was not significantly linked to the \textit{Prsq4-5} region in the D2 F2 or N2 crosses (data not shown).

\textit{Prsq6} is linked to \textit{D12Mit5} near 82 Mb (one-LOD support interval, 19–105 Mb) on chromosome 12 in the FVB × BALB backcross (\(P < 0.046, \text{Table 2}\)). The FVB allele is the susceptible allele (1.9-fold effect; Table 2). \textit{Prsq6} was not identified in the F2 analysis; any effect may be obscured by the \textit{Prsq2} and \textit{Prsq5} effects.

\textbf{Haplotype analysis for the \textit{Prsq1-2} and \textit{Prsq4-5} intervals}

We performed a preliminary analysis of the single-nucleotide polymorphisms (SNP) carried by the FVB, B6, and BALB strains within the combined one-LOD support intervals for \textit{Prsq1-2} and \textit{Prsq4-5} using data from the Mouse HapMap Imputed Genotype Resource (Supplementary Fig. S1; ref. 29). These broad intervals (~60–70 Mb each) contain large numbers of genes (1,011 and 977 for chromosomes 2 and 4, respectively). B6 and BALB carry alleles on chromosome 2 that confer susceptibility relative to FVB; on chromosome 4, B6 and BALB carry alleles with opposite effects. Therefore, the simplest models for the haplotype patterns among these strains are that B6 and BALB share haplotypes distinct from that of FVB on chromosome 2 (\textit{Prsq1-2}) and that FVB, B6, and BALB all differ from one another in haplotype on chromosome 4 (\textit{Prsq4-5}). Under these models, the numbers of potential candidates for the susceptibility loci...
are limited to 126 and 287 genes (Supplementary Table S4) for Pry1-2 and Pry4-5, respectively. Under the hypothesis that each locus is unique (e.g., the B6 allele of Pry1 differs from both the FVB and BALB alleles, which may be the same), the number of candidates within the one-LOD support intervals for Pry1, Pry2, Pry4, and Pry5 includes 654, 164, 138, and 535 genes, respectively.

Inbred B6 and BALB mice consomic for the Ela-KRASG12D transgenic Y chromosome are more susceptible than FVB

The Ela-KRASG12D transgene in this mouse model is located on the Y chromosome. By repeated backcrossing B6FVB-Tg(Ela-KRASG12D)F1 to B6 mice, and BALBFVB-Tg(Ela-KRASG12D)F1 to BALB mice, we have developed mice that are consomic for the FVB Y chromosome carrying the Ela-KRASG12D transgene on either a B6 or BALB genetic background (n ≥ 10). Analysis of B6-Chr YFVB-Tg(Ela-KRASG12D) and BALB-Chr YFVB-Tg(Ela-KRASG12D) consomic mice at 12 months of age revealed the development of a significantly larger number of lesions relative to FVB-Tg(Ela-KRASG12D) mice (Table 3). We found a 4-fold increase in mean lesion multiplicity in B6-Chr YFVB-Tg(Ela-KRASG12D) (11.1 ± 6.4) and a 10-fold increased effect in BALB-Chr YFVB-Tg(Ela-KRASG12D) (28 ± 13) relative to FVB-Tg(Ela-KRASG12D) (2.8 ± 1.6).

Histologic analysis of B6-Chr YFVB-Tg(Ela-KRASG12D) and BALB-Chr YFVB-Tg(Ela-KRASG12D) consomic mice revealed ducts containing irregular epithelial cells similar to those identified in the FVB-Tg(Ela-KRASG12D) mice (16). Focal carcinomas of mixed morphologic differentiation (acinar and/or ductal characteristics) were identified in ~13% of FVB-Tg(Ela-KRASG12D) (4 of 32 mice), ~22% of B6-Chr YFVB-Tg(Ela-KRASG12D) (7 of 32 mice), and ~60% of BALB-Chr YFVB-Tg(Ela-KRASG12D) (18 of 30 mice). Mouse PanINs (mPanIN; ref. 11) have projections of papillary epithelium extend into the lumens of the PanIN2 lesion from B6-Chr YFVB-Tg(Ela-KRASG12D) mouse. C, invasive adenocarcinoma in BALB-Chr YFVB-Tg(Ela-KRASG12D) mouse. Scale bars, 20 μm (A and B) and 100 μm (C).

Figure 2. Lesions in B6 and BALB transgenic mice are more advanced than those in FVB. Paraffin-embedded pancreatic tissue sections were mounted on a slide and stained with H&E. A, normal duct in FVB-Tg(Ela-KRASG12D) mouse. B, projections of papillary epithelium extend into the lumens of mPanIN2 lesion from B6-Chr YFVB-Tg(Ela-KRASG12D) mouse. C, invasive adenocarcinoma in BALB-Chr YFVB-Tg(Ela-KRASG12D) mouse. Scale bars, 20 μm (A and B) and 100 μm (C).

and β-actin mRNA from six, seven, or nine animals (B6, BALB, and FVB, respectively) were quantitated, and the expression of KRAS/Kras mRNA relative to β-actin was determined. KRAS/Kras expression in B6 and BALB did not differ significantly from FVB (P > 0.42, B6 versus FVB; P > 0.37, BALB versus FVB; Supplementary Fig. S2). RT-PCR analysis using RNA isolated immediately from fresh tissue revealed similar levels of transgenic (human) KRAS mRNA for FVB-Tg(Ela-KRASG12D) and BALB-Chr YFVB-Tg(Ela-KRASG12D) consomic mice. The expression of the transgenic KRAS mRNA relative to the geometric mean of Sdha, Ywhaz, and Actg1 was 0.039 ± 0.030 for FVB-Tg(Ela-KRASG12D) and 0.028 ± 0.001 for BALB-Chr YFVB-Tg(Ela-KRASG12D) consomic mice. In addition, there was a <2-fold difference in Kras expression between FVB-Tg(Ela-KRASG12D) and FVB-Chr YFVB-Tg(Ela-KRASG12D) F1 hybrid animals. Consistent with these findings, we were not able to detect differences in Kras mRNA levels among F1 progeny (data not shown). Human KRAS expression was not detected in nontransgenic mice.

Similarly, we did not detect a significant difference in levels of Ras or phospho-MAPK protein expression across strains by Western blot (P > 0.18; Fig. 3). The relative amounts of the p42 and p44 isoforms were variable between experiments and did not correlate with susceptibility. Consistent with these findings among consomics, we were not able to detect differences in phospho-MAPK expression among F1 progeny (data not shown).

KRAS/Kras mRNA and protein levels are similar in FVB, B6, and BALB strains

RT-PCR analysis using RNA from frozen tissue revealed similar levels for the total amount of KRAS/Kras mRNA (from transgenic human and endogenous mouse genes) across the FVB-Tg(Ela-KRASG12D), B6-Chr YFVB-Tg(Ela-KRASG12D), and BALB-Chr YFVB-Tg(Ela-KRASG12D) consomic strains. KRAS/Kras

3 Unpublished data.

Website: www.aacrjournals.org Cancer Res; 70(21) November 1, 2010 8403

Published OnlineFirst October 19, 2010; DOI: 10.1158/0008-5472.CAN-09-3980

Downloaded from cancerres.aacrjournals.org on April 14, 2017. © 2010 American Association for Cancer Research.
Discussion

Linkage analysis identified Prsq1-3, Prsq4-5, and Prsq6 on chromosomes 2, 4, and 12, respectively, as the predominant modifiers of lesion multiplicity in Ela-KRAS\textsuperscript{G12D} mice. The Prsq1-3 loci on chromosome 2 in B6, BALB, and D2 mice confer susceptibility relative to FVB. The B6 allele of Prsq1 is semidominant. Dominance of the BALB allele of Prsq2, which had no significant effect in the intercross, could not be assessed. The D2 allele of Prsq3 is recessive. Prsq4 and Prsq5 on chromosome 4 have opposite effects in B6 and BALB relative to FVB. The B6 allele of Prsq4 is semidominant and confers resistance, whereas the BALB allele of Prsq5 is recessive and confers susceptibility. Prsq6 was found on chromosome 12; the BALB allele conferred resistance in the backcross only. The effects of Prsq1-6 are unlikely to be due to differences in transgene expression: RT-PCR and Western blot analysis determined that levels of transgene expression are similar among the inbred consomic strains and the backcross and intercross progeny.

More detailed analysis may reveal multiple modifier genes within the Prsq loci. The identification of multiple modifiers within QTLs has been documented in intestinal and breast cancer and plasmacytoma, where multiple syntenic genetic loci on chromosome 2 in B6, BALB, and D2 mice contribute to the number of genes that underlie these QTLs. Prsq3 and Prsq4 together account for all of the phenotype of B6 consomic mice relative to FVB. BALB-Chr Y\textsuperscript{FVB-Tg(Ela-KRAS\textsuperscript{G12D})} consomic mice developed 11.8 ± 6.4 lesions, 4-fold more than FVB-Tg(Ela-KRAS\textsuperscript{G12D}) mice. The lesion multiplicity of Prsq4\textsuperscript{B6/B6} and Prsq5\textsuperscript{B6/B6} mice in the F2 cross was 11.8 ± 2.6. This similarity suggests that Prsq1 and Prsq4 together account for the susceptibility of BALB consomic mice relative to FVB. The consomic strains will be helpful in the analysis of congenic strains carrying Prsq loci on B6 or BALB backgrounds. In addition, their development of invasive lesions at a relatively young age (12 months) should make the BALB consomics useful in the study of more advanced stages of pancreatic cancer.

Haplotype analysis identified many genes consistent with the simplest models for the chromosome 2 and 4 loci. The 126 genes on chromosome 2 that are candidates for both Prsq1 and Prsq2 include 11 involved in transcription, 10 involved in development, and 9 involved in cell differentiation. These candidates include the TGF\textsuperscript{β} family member Bmp2, the Rb family member p107, C/EBP, and the Notch ligand gene jagged 1. The 287 genes on chromosome 4 that are candidates for both Prsq4 and Prsq5 include 23 involved in transcription, 21 involved in development, and 16 involved in signal transduction. These chromosome 4 candidates include Deleted in Bladder Cancer 1, Myc, and the Ras gene family member Rab3b. Gene expression and mapping analysis of congenic lines carrying defined portions of chromosomes 2 and 4 will be needed to reduce the large numbers of candidates for each locus and allow testing by transgenesis or gene replacement.

Analysis of the genomic locations of the Prsq loci also revealed candidates that have already been implicated as having effects on pancreatic tumorigenesis (3, 8, 12, 33–36). One possible Prsq1-3 candidate gene near 172 Mb on chromosome 2, within the one-LOD support intervals for Prsq1 and Prsq3, is Aurka. The B6, BALB, and D2 strains share a SNP haplotype for this gene that differs from that for FVB. Overexpression of an oncogenic version of Aurka causes mild pancreatic duct dysplasia (33). However, expression of Aurka mRNA relative to FVB (set at 1.0) among susceptible strains carrying the KRAS transgene varied and was 3.6 for B6 and 0.8 for BALB (data not shown). Although these results do not support Aurka as a candidate for Prsq2, they are consistent with the possibility that elevated Aurka expression in B6 accounts for some of the Prsq1 effect.

Two candidates for the Prsq4-5 loci on mouse chromosome 4 (peak LOD\textsubscript{p} score at 63.0 Mb) are Tgfbr1 and Cdkn2a, at 47.4 and 88.9 Mb, respectively. Alterations in CDKN2A/Cdkn2a and
TGFβ1/Tgfb1 signaling have been found to have an effect on pancreatic cancer progression in mice and also have been observed in human PDAC (3, 8, 12, 34–36). Tgfb1 is well outside the one-LOD support intervals for Prsq4–5. However, because of the strong data supporting a role for transforming growth factor-β (TGF-β) signaling in pancreatic cancer in both humans and mice, D4Mit6 (500 Mb) was used to assess directly linkage of the more proximal modifier, Prsq4, to Tgfb1 (47.4 Mb) in B6FVB-Tg(Ela-KRASG12D)F2 mice. Results were compared with those for the most significant marker for Prsq4, where there is a 2.9-fold effect of B6 alleles. There was no effect at D4Mit6 near Tgfb1, indicating that this gene is not likely to be Prsq4.

Several observations make Cdkn2a an appealing candidate for Prsq5. First, Cdkn2a is near the midpoint of the interval between D4Mit17 (63 Mb) and D4Mit31 (107 Mb), both of which yielded high LODv scores (2.84 and 2.45, respectively) in the BALB × FVB F2 cross. Second, we have observed that Ela-KRAS transgenic mice heterozygous for p16-null mutations develop pancreatic ductal tumors with greatly reduced latency and frequent metastasis relative to p16 wild-type mice. Finally, the BALB allele of Cdkn2a has been shown to be hemizygous for p16 function (32, 37). Analysis of SNPs within the Cdkn2a gene (29) indicates that the BALB haplotype is unique among the four strains we studied; FVB and B6 mice have identical SNP haplotypes for this gene.

Prsq1–3 and Prsq4–5 correspond to two of the human chromosomal regions that are most frequently gained or lost in pancreatic cancer (38, 39). Part of the region of peak linkage for Prsq1–3 (152–179 Mb) on chromosome 2 corresponds to human 20q11-13, which carries AURKA and is amplified in approximately half of pancreatic cancers (38, 39). Human chromosome 9p, which carries CDKN2A and corresponds to part of the Prsq4–5 region on chromosome 4, is lost in approximately half of pancreatic cancers (38, 39).

The close correspondence between predominant human chromosomal changes and the Prsq loci indicates that further study of the mouse modifiers is likely to help explain the development of pancreatic cancer in humans. The development of congenic lines for the Prsq loci will allow investigation of biological mechanisms and the roles they play in pancreatic cancer progression. Additionally, such lines will allow the individual and additive effects of the loci to be analyzed, provide a framework for detailed physical mapping, and, ultimately, facilitate positional cloning of genes within these regions. The molecular identification of modifier genes in the mouse model should facilitate the search for human susceptibility genes and the identification of populations that might be predisposed to PDAC.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank Rebecca Baus and the animal care staff in McArdle, Medical Science Center, and the Biotron for their work with the mice; Meg Bowden and Adam Jochem for technical assistance; Melissa Schutten for her advice on histopathology; and Dr. William F. Dove for his critical comments on the manuscript.

Grant Support

NIH grants P01CA022484 (N.R. Drinkwater) and R01CA076361 (E.P. Sandgren). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received 10/28/2009; revised 07/08/2010; accepted 07/22/2010; published OnlineFirst 10/19/2010.

References


M. Schutten and E.P. Sandgren, unpublished data.


Identification of Susceptibility Loci in a Mouse Model of KRAS G12D-Driven Pancreatic Cancer

Tonia C. Jorgenson, Bret R. Williams, Allyson Wendland, et al.

Cancer Res  Published OnlineFirst October 19, 2010.

Updated version  Access the most recent version of this article at: doi:10.1158/0008-5472.CAN-09-3980

Supplementary Material  Access the most recent supplemental material at: http://cancerres.aacrjournals.org/content/suppl/2010/10/18/0008-5472.CAN-09-3980.DC1

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.