Aberrant recombination involving the granzyme locus occurs in Atm<sup>−/−</sup> T-cell lymphomas

Christopher J. Winrow<sup>1</sup>, Daniel G. Pankratz<sup>1</sup>, Cecile Rose T. Vibat<sup>2</sup>, T.J. Bowen<sup>3</sup>, Marie A. Callahan<sup>2</sup>, Amy J. Warren<sup>2</sup>, Brian S. Hilbush<sup>2</sup>, Anthony Wynshaw-Boris<sup>3</sup>, Karl W. Hasel<sup>2</sup>, Zoë Weaver<sup>4</sup>, David J. Lockhart<sup>1,5</sup> and Carrolee Barlow<sup>1,6,*</sup>

<sup>1</sup>The Salk Institute for Biological Studies, The Laboratory of Genetics, 10010 North Torrey Pines Road, La Jolla, CA 92037, USA, <sup>2</sup>Digital Gene Technologies, Inc., 11149 North Torrey Pines Road, La Jolla, CA 92037, USA, <sup>3</sup>Departments of Pediatrics and Medicine, School of Medicine, University of California, San Diego, La Jolla, CA 92093-0627, USA, <sup>4</sup>Avalon Pharmaceuticals, Inc., 19 Firstfield Road, Gaithersburg, MD 20878, USA, <sup>5</sup>Ambit Biosciences, 4215 Sorrento Valley Boulevard, San Diego, CA 92121, USA and <sup>6</sup>Brain Cells, Inc., 10835 Road to the Cure, San Diego, CA 92121, USA

Received May 16, 2005; Revised July 10, 2005; Accepted August 1, 2005

Ataxia telangiectasia (A-T) is an autosomal recessive disease caused by loss of function of the serine/threonine protein kinase ATM (ataxia telangiectasia mutated). A-T patients have a 250–700-fold increased risk of developing lymphomas and leukemias which are typically highly invasive and proliferative. In addition, a subset of adult acute lymphoblastic leukemias and aggressive B-cell chronic lymphocytic leukemias that occur in the general population show loss of heterozygosity for ATM. To define the specific role of ATM in lymphomagenesis, we studied T-cell lymphomas isolated from mice with mutations in ATM and/or p53 using cytogenetic analysis and mRNA transcriptional profiling. The analyses identified genes misregulated as a consequence of the amplifications, deletions and translocation events arising as a result of ATM loss. A specific recurrent disruption of the granzyme gene family locus was identified resulting in an aberrant granzyme B/C fusion product. The combined application of cytogenetic and gene expression approaches identified specific loci and genes that define the pathway of initiation and progression of lymphoreticular malignancies in the absence of ATM.

INTRODUCTION

The transformation of a normal cell to a tumor cell depends in part on mutations in genes that control the cell cycle. Cell-cycle regulation plays a major role in maintaining the integrity of the genome. Defects in checkpoint control contribute to genomic alterations such as deletions, translocations and amplifications that commonly occur during the evolution of a normal cell to a cancer cell. ATM (ataxia telangiectasia mutated) is a key protein responsible for arresting the cell cycle in response to DNA damage and has a role in genetic stability and cancer susceptibility. Nearly one-third of ataxia telangiectasia (A-T) patients develop aggressive and invasive forms of either lymphocytic leukemia or non-Hodgkin lymphomas (1,2). A mouse model of A-T (Atm<sup>−/−</sup>) closely mimics the human condition and has been useful for defining the role of ATM in cancer (3,4). All Atm<sup>−/−</sup> mice succumb to aggressive T-cell lymphoblastic lymphoma early in life, and these tumors closely resemble those found in A-T patients in several respects: (1) the tumors develop in very young Atm<sup>−/−</sup> mice, similar to the lymphoreticular cancers in A-T patients which arise in childhood (3–7); (2) the tumors are highly proliferative and invasive (1,3,8–10); (3) virtually all tumors found in Atm<sup>−/−</sup> mice and the majority of T-cell leukemias in A-T patients are CD4<sup>+</sup>/CD8<sup>+</sup> (3,5–7,11–13); and (4) tumors from Atm<sup>−/−</sup> mice

*To whom correspondence should be addressed. Tel: +1 8588127615; Fax: +1 8588127630; Email: cbarlow@braincellsinc.com

© The Author 2005. Published by Oxford University Press. All rights reserved.

The online version of this article has been published under an open access model. Users are entitled to use, reproduce, disseminate, or display the open access version of this article for non-commercial purposes provided that: the original authorship is properly and fully attributed; the Journal and Oxford University Press are attributed as the original place of publication with the correct citation details given; if an article is subsequently reproduced or disseminated not in its entirety but only in part or as a derivative work this must be clearly indicated. For commercial re-use, please contact: journals.permissions@oupjournals.org
contain translocations of chromosomes 12 and 14 in regions homologous to translocated regions of human chromosome 14 in A-T patients (3,4,14). These regions are also frequently mutated in other human hematopoietic cancers that occur in patients that do not have A-T (15–17).

On the basis of cytogenetic data and the role of ATM in DNA repair following ionizing radiation (IR), it is likely that lymphoblastoid cancers arising in the absence of ATM are due to specific translocations at loci that undergo V(D)J recombination. In support of such an hypothesis is the finding that human chromosome 14q11.2 harbors the T-cell receptor alpha (TCRα) gene and abnormalities in this region have been detected in A-T patients (18–24) and in Atm−/− mice (4). Recombination of IR and V(D)J produces DNA double-strand breaks (DSBs) that are repaired by non-homologous end joining (NHEJ), and ATM is thought to activate proteins involved in NHEJ in response to IR. This has lead to the suggestion that defective V(D)J recombination due to loss of ATM function is responsible for tumor formation. However, recently we showed that V(D)J recombination is not required for lymphoma formation in the absence of Atm, as Atm−/− mice deficient in Rag1 or Rag2 (and therefore incapable of V(D)J recombination) still succumb to T-cell lymphomas (TCLs) (14,25). An alternative possibility is that following loss of ATM, the development of tumors occurs as a result of disruptions in signaling to the cell-cycle checkpoint machinery. For example, in response to IR, ATM activates p53 (26–28), and mutations that render p53 inactive are the most frequent cause of cancers in humans (29–31). Regardless of a link between defective ATM signaling to p53 and cancer, the pattern of tumor development and chromosomal abnormalities that occur in the absence of ATM in humans and mice suggests that there is a requirement for specific chromosomal regions to undergo aberrant recombination and that these rearrangements result in molecular changes that promote tumor formation and invasion (4,24).

To identify the molecular events responsible for tumorigenesis in the absence of ATM, it is necessary to identify the loci and the genes affected by the conserved rearrangements observed in these tumors. In this paper, we show that Atm−/− TCLs are unique from p53−/− lymphomas both cytogenetically and at the level of gene expression, establishing that the loss of the p53 cell-cycle checkpoint is not responsible for lymphoma formation in the absence of ATM. Further, by coupling cytogenetic analyses of Atm−/− TCLs with gene expression profiling we identified recurrent aberrations on chromosome 14 within the granzyme (Gzm) family locus at specific sites of homology between family members. The disruptions at this locus result in the rearrangement and inappropriate expression of a granzyme protein involved in cancer promotion and invasion and suggest a mechanism for tumor promotion in the absence of ATM involving aberrant homologous recombination (HR).

RESULTS

Characteristic translocations in Atm−/− TCLs are dependent on normal p53 alleles

Atm−/− mice develop CD4−/CD8+ (immature) TCLs with characteristic cytogenetic abnormalities consisting of inter- and intra-chromosomal rearrangements of chromosomes 12 and 14 and amplification of chromosome 15 (Table 1) (3,4,14,25). Although p53 and ATM cooperate in response to DNA damage and regulating the cell cycle, tumors which arise as a result of deficiencies of p53 and ATM are markedly different (Fig. 1A and Table 1) (4,32–35). For example, Atm−/− mice rapidly develop TCLs, whereas p53−/− mice show more latent development of a variety of different tumors including TCLs. Atm−/− TCLs are more immature (CD4+/CD8+) than p53−/− TCLs (frequently CD3+) (see Table 1). Atm−/− TCLs have inter- and intra-chromosomal aberrations including translocations involving chromosomes 12 and 14 (4), whereas TCLs from p53−/− mice exhibit aneuploidy with only rare translocations (for example, see Table 1 and Fig. 1E) (30,36–39). Interestingly, both Atm−/− and p53−/− tumors have amplifications of chromosome 15 (4,30,39,40).

To identify Atm-specific lesions and how p53 loss may impact the cytogenetic and molecular events underlying lymphomagenesis, mice deficient in Atm and lacking one or both alleles of p53 were generated, and the TCLs that developed were isolated and studied by spectral karyotyping (SKY) and flow cytometry. SKY was done on TCLs at early passage and the karyotypes were compared with those from Atm−/− mice. Four of the five TCLs isolated from Atm−/−p53−/+ mice harbored translocations involving chromosomes 12 and 14 but aneuploidy was markedly increased (Table 1 and Fig. 1A–C), suggesting that haplo-insufficiency of p53 during tumor formation results in a combined phenotype possibly due to a shared ATM pathway. Mice lacking Atm and both alleles of p53 (Atm−/−p53−/−) rapidly succumb to a host of tumors including both B- and T-cell lymphomas within the first weeks of life (41) (data not shown). TCLs from Atm−/−p53−/− mice were more mature (CD3+) and exhibited both increased aneuploidy and translocations, which no longer consistently involved chromosome 12 or 14 (Fig. 1D and Table 1). Notably, abnormalities of chromosome 12 were rarely found. Therefore, not only is the clinical pathology of Atm−/− TCLs different from p53−/− TCLs, but the two tumor types also show distinct developmental differences and unique chromosomal disruptions.

Global gene expression patterns highlight the unique nature of Atm−/− lymphomas

Recently, global expression profiling has proven extremely useful in identifying subtypes of leukemias and lymphomas (42–46). To test if a similar approach could be used to study ATM-specific lymphomagenesis, cDNA microarrays and Total Gene Expression Analysis (TOGA®) were used (47–50). First, cDNA microarrays were used to identify genes differentially expressed in Atm−/− thymus as compared to wild-type thymus prior to tumor formation (Fig. 2A). Next, samples from wild-type thymus were compared with two independent p53−/− TCLs and four Atm−/− TCLs (AT-4, AT-7, AT-12 and AT-13) using cDNA microarrays (Supplementary Material, Tables S3–S5 for gene lists and Supplemental Methods for experimental design and analysis criteria). In comparisons between the Atm−/− TCLs and wild-type thymus, 154 genes were identified as differentially expressed (green circle in Fig. 2B and Supplementary Material, Tables S3 and S4). A similar comparison between
Atm genes were misexpressed in both TCLs and wild-type thymus identified 300 genes as differentially expressed (blue circle in Fig. 2B and Supplementary Material, Table S5). This data demonstrated that the p53−/− TCLs were more different from normal thymus (205 of 300 genes, or 68%) than Atm−/− TCLs (62 of 154, or 40%) when analyzed using similar criteria. Ninety-one genes were not only abnormally expressed in all these 91 genes were not only abnormally expressed in all TCLs (62 of 154, or 40%) when analyzed using similar criteria. Ninety-one genes were not only abnormally expressed in all TCLs, and this requires an intact p53 allele.

The conserved disruption of chromosome 14 involves the granzyme gene family

Chromosomes 12, 14 and 15 are consistently disrupted in Atm−/− TCLs (Table 1). Although cDNA microarrays were informative in terms of identifying gene pathways that may be involved in lymphomagenesis and survival, these experiments did not identify specific loci affected by translocations and gene fusion events. Sixty-two genes were differentially expressed exclusively in Atm−/− TCLs. Four of these genes were localized to chromosome 14, four to chromosome 15 and two to chromosome 12 (Fig. 2B and Supplementary Material, Table S3). Three genes on chromosome 14 were upregulated. These were cam kinase II beta, an EST (AA198542) and glia maturation factor. One gene was downregulated, TGF-beta-1-induced transcript. The two genes on chromosome 12, secreted modular calcium BP1 and Ena-vasodilator-stimulated phosphoprotein (Evl), were downregulated. Evl lies between the Tcl1 and IgH loci on chromosome 12. The four genes that mapped to chromosome 15, ATP synthetase H+ transporting mitochondrial F1 complex subunit C, proline-rich protein, Map3k12 and cytosolic aminopeptidase P, were all upregulated consistent with the cytogenetic network of chromosome 15 amplification. We combined data from cDNA microarrays with TOGA® to identify additional genes impacted by the conserved chromosome lesions affecting chromosomes 12, 14 and 15 in Atm−/− TCLs. Three Atm−/− TCLs with similar cytogenetic profiles (AT-4, AT-7 and AT-13) were compared with one TCL which had several unique chromosomal aberrations (AT-12) (Table 1).

Experiments using TOGA® identified 13 569 transcripts in AT-4, 12 999 in AT-7, 13 111 in AT-12 and 13 398 in AT-13. The expression profile of each tumor line was then compared among the four. One thousand and fifty-two transcripts were differentially expressed at ≥2-fold between any one of the four Atm−/− TCLs. Next, we identified genes exhibiting unique or conserved patterns of expression between the tumor cell lines (see Materials and Methods). Twenty-four genes were identified with increased expression in a single cell line (consistent with an activating translocation, Table 2), 30 genes with decreased expression in a single cell line (consistent with a deletion, Table 2) and nine genes with increased expression in two cell lines (consistent with an activating translocation, Table 2). Two genes with equal

Table 1. Translocations at chromosomes 12 and 14 are unique to Atm−/− TCLs, and this requires an intact p53 allele

<table>
<thead>
<tr>
<th>Tumor</th>
<th>Genotype</th>
<th>Conserved</th>
<th>Unique</th>
<th>CD4</th>
<th>CD8</th>
<th>CD3</th>
</tr>
</thead>
<tbody>
<tr>
<td>AT-4b</td>
<td>Atm−/−</td>
<td>Del (12), +15, Del (14), T(12;14)</td>
<td>2n; Dp(11), T(14;X), T(X;11), T(14;15)b</td>
<td>+</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>AT-7b</td>
<td>Atm−/−</td>
<td>Del (12), +15, Del (14), T(12;14)</td>
<td>2n; T(14;16), Is (14;1)</td>
<td>+</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>AT-12b</td>
<td>Atm−/−</td>
<td>Del (12), +15, Del (14), T(12;14)</td>
<td>2n; T(12;9), T(9;15), +10, +13</td>
<td>+</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>AT-13b</td>
<td>Atm−/−</td>
<td>Del (12), +15, Del (14), T(12;14)</td>
<td>2n; Ins(14;15), T(14;15)b</td>
<td>+</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>AT-10</td>
<td>Atm−/−</td>
<td>Del (12), +15</td>
<td>2n; T(12;10), Dpl4, T(X;15), Del(X), T(17;1), Dp (1), Del (16), −11</td>
<td>+</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>AT-11</td>
<td>Atm−/−</td>
<td>Del (12), +15</td>
<td>2n; T(12;6), Dp(6), Dic(14;14), Dp(14), Dic(15;15), Rb(16;16), Dp(16)</td>
<td>+</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>APT-2</td>
<td>Atm−/−</td>
<td>p53+/−</td>
<td>+15, T (12;14)</td>
<td>2n; Rb(10.10), T(15;X), Dp(14)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>APT-3</td>
<td>Atm−/−</td>
<td>p53+/−</td>
<td>+15, T (12;14)</td>
<td>2n; T(14;2), T(5;14;2), T(8;6)</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>APT-4</td>
<td>Atm−/−</td>
<td>p53+/−</td>
<td>+15, T(12;14)</td>
<td>2n; T(4;14), T(14;2), Dic(2;14)</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>APT-5</td>
<td>Atm−/−</td>
<td>p53+/−</td>
<td>+15</td>
<td>2n and 3n clones; Rb(9.Del(15)), +5, −13, +15</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>292-3</td>
<td>Atm−/−</td>
<td>p53+/−</td>
<td>+15</td>
<td>2n; T(17;6); +14</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>101-3</td>
<td>Atm−/−</td>
<td>p53+/−</td>
<td>+15</td>
<td>Hyperdiploid and 4n clones, Dp(14), -8</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>p21-1</td>
<td>Atm−/−</td>
<td>p21+/−</td>
<td>+15</td>
<td>T(12;9)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>p53-1</td>
<td>Atm−/−</td>
<td>p53+/−</td>
<td>+15</td>
<td>+, +, +, +, +1, +11, +14</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

*aOnly the most frequently observed aberrations, and therefore individual karyotypes may contain additional inconsistent aberrations. Abbreviations: translocation (T), deletion (Del), duplication (Dp), insertion (Is), dicentric chromosome (Dic), Robertsonian translocation (Rb). Aberrations were defined using the nomenclature rules from the Committee on Standardized Genetic Nomenclature for Mice (www.informatics.jax.org). *bTumor lines profiled by TOGA™. Bold names indicate tumor lines examined by northern analysis.
Figure 1. *Atm*<sup>−/−</sup> TCLs exhibit translocations, whereas *p53*<sup>−/−</sup> TCLs are aneuploid. (A–C) A representative *Atm*<sup>−/−</sup> *p53*<sup>+/−</sup> TCL (APT-4) that exhibits several translocations involving chromosome 14. (A) SKY classification of a metaphase from APT-4. The display colors are shown on the left for each chromosome, next to the inverted DAPI image and the spectra-based classification colors. The full karyotype for this metaphase is 40,X, Dic(Del(2);T(4;14)), T(4;14), Del(7), T(12;14), T(14;2), Dp(14), T(Dp(16);3), +Del(12), +15, −19, −Y. (B) Unclassified metaphase from (A) in hybridization display colors. Translocations are visible as 2-color chromosomes. The arrow indicates a T(4;14). (C) FISH analysis of APT-4 with BAC probes for the TCRα locus on chromosome 14. The chromosomes are counterstained with DAPI for visualization (blue). Hybridization with a TcrV<sub>α</sub> variable region probe (green, arrow) shows that several duplications of this region (cytogenetic band 14D1–D2) have occurred, comparable to aberrations seen in *Atm*<sup>−/−</sup> *p53*<sup>+/−</sup> TCLs. Hybridization with a TcrC<sub>α</sub> constant region probe (red, arrowheads) reveals that none of the signals colocalize with the TcrV<sub>α</sub> probe, consistent with the translocations seen by SKY. (D) SKY of an *Atm*<sup>−/−</sup> *p53*<sup>−/−</sup> TCL (101-3; 4n clone). Translocations involving chromosomes 1, 5 and 14 are shown in their classification colors next to the display colors. Note that the tumor is aneuploid as well. The karyotype is 85,XX, T(1;5), T(5;1), Dp(14), T(14;3), +4 × 2, −6, +10 × 2, −11 × 2, +12, +14, +15 × 5, −19, −Y × 2. (E) SKY analysis of a *p53*<sup>−/−</sup> TCL (p53-1). This tumor exhibits aneuploidy but has no structural aberrations. The karyotype is 47,XX, +1, +4, +5, +11, +14 × 2, +15.
expression levels in all tumor cell lines were also evaluated as controls (Table 2). These 65 candidates were sequenced and compared with the GenBank database. Of the 65 candidates isolated, 39 were known genes, 11 were uncharacterized murine homologs, 12 were ESTs and three were completely novel genes not present in GenBank (see Table 2). Follow-up validation by northern analysis was done on 25 randomly selected candidate genes. Northern analysis was consistent with TOGA® for 17 genes, five had undetectable signals, two cross-reacted with other family members precluding a definitive analysis and one showed a different pattern on northern as compared with TOGA®. One candidate that did not confirm represented a mitochondrial-encoded transcript. Therefore, the pattern of expression obtained by TOGA® for 17 of the 18 transcripts was confirmed by independent northern blot analysis.

Several genes were identified that were overexpressed and which reside on chromosome 15. But many of these genes were also differentially expressed in p53−/− TCLs and so were not ATM-specific or had no clear role in TCL formation (see Table 2 and Supplementary Material, Tables S3–S5 for details of fold changes and chromosomal locations). Only one gene was identified as abnormally expressed that resides on chromosome 12 near the interrupted region (Evi), and this gene was downregulated (Table 2, Fig. 3C and Supplementary Material, Table S4), which is unlikely to be associated with an activating translocation. In contrast, we identified the GzmC gene tag as upregulated by TOGA® in two of the four TCLs (Table 2). Northern analysis using an EST probe for GzmC showed that GzmC was detectable in four of the seven Atm−/− TCLs (Fig. 3B) but was not expressed in tumors lacking p53. In addition, a second abnormally large band was detected in several Atm−/− TCLs suggesting that not only GzmC was overexpressed, but also an aberrant form of the message was produced in Atm−/− TCLs consistent with an activating translocation event. GzmC belongs to a family of closely related granzymes clustered on chromosome 14 at 20.5 cM (14qC1–C2) in mice and 14q11.2 in humans (51–57). The GzmC locus is very close to the TCRα locus on chromosome 14 at 19.5–19.7 cM (14qC1–C2) (Fig. 3A). This is the region consistently observed to be disrupted in Atm−/− TCLs in both mice and humans (4,18–24). To investigate if the GzmC locus was involved in Atm−/− translocations, FISH was done using genomic probes for GzmC (chromosome 14), TCRα (chromosome 14) and Tcl1 (chromosome 12) (Fig. 3C). TCRα was used to pinpoint the region of chromosome 14 corresponding to 19.5–19.7 cM (14qC1–C2) (Fig. 3A). Tcl1 was used to determine the region corresponding to 52.0 cM on chromosome 12 (58–61). We have previously shown that there are translocations involving chromosome 14 and regions of chromosome 12 that are near the Tcl1 locus, although Tcl1 is not consistently involved nor is there expression of Tcl1 in any Atm−/− TCL (4). Examination of wild-type cells confirmed that the GzmC and TCRα loci are in close proximity (upper color panel in Fig. 3C, and data not shown). FISH analysis of AT-7 TCLs showed that the GzmC locus was duplicated and inverted on chromosome 14, and an additional copy of the gene is on the portion of chromosome 14 that was translocated to chromosome 12 (see Fig. 3C). Interestingly, although the GzmC locus was

Figure 2. Gene expression patterns of Atm−/− TCLs are unique from pre-malignant Atm−/− thymocytes and p53−/− TCLs. (A) The 31 genes identified at various time points that were upregulated (red) or downregulated (green) in Atm−/− thymus in comparison to age-matched wild-type controls. The X-axis indicates age in weeks (4, 5, 8, 9 and 16) and the Y-axis the average FC for the replicate experiment at each time point. Both CD53 and Igo are represented more than once on the arrays and the average FC ranges for those values are shown. The names of genes are listed below the panel. (B) Venn diagram showing the number of genes differentially expressed in Atm−/− TCLs (green circle), Atm−/− thymus (black circle) and p53−/− TCLs (blue circle), when compared with wild-type thymus. Those genes differentially expressed in the same direction are listed in (A) and colored as follows: Atm−/− thymus and Atm−/− TCLs (1 gene, magenta), Atm−/− thymus and p53−/− TCLs (4 genes, blue) or all three conditions (3 genes, green); genes commonly differentially expressed in both Atm−/− and p53−/− TCLs as compared to wild-type thymus (91 genes) are listed in Supplementary Material, Table S5. The number in parentheses indicates the number of genes differentially expressed in opposite directions (Fig. 3A, orange); these genes are all upregulated in Atm−/− thymus (Fig. 3A); Genes differentially expressed exclusively in all Atm−/− TCLs compared with normal thymus (62 transcripts, Supplementary Material, Table S4) and all genes different in p53−/− TCLs (300 transcripts, Supplementary Material, Table S6) are also shown.
Table 2. Genes exhibiting unique or conserved patterns of expression between the tumor cell lines

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Accession number</th>
<th>Tumor line</th>
<th>Mouse</th>
<th>Human</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Genes with increased expression in a single tumor cell line</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RhoC (confirmed)</td>
<td>X80638</td>
<td>AT4</td>
<td>3</td>
<td>1k21–p13</td>
</tr>
<tr>
<td>Homology to human KIAA0439</td>
<td>AB007899</td>
<td>AT7</td>
<td>3; 41.5 cm</td>
<td>1k22</td>
</tr>
<tr>
<td>Mouse p162/centrosin/EIF2</td>
<td>U14172</td>
<td>AT7</td>
<td>19</td>
<td>10q26</td>
</tr>
<tr>
<td>Mouse transglutaminase 2</td>
<td>M55154</td>
<td>AT7</td>
<td>2; 89.0 cm</td>
<td>20q12</td>
</tr>
<tr>
<td>Homology to human P53BP2</td>
<td>U09582</td>
<td>AT7</td>
<td>1</td>
<td>1q42.1</td>
</tr>
<tr>
<td>EST</td>
<td>AW060549</td>
<td>AT7</td>
<td>n/a</td>
<td>14</td>
</tr>
<tr>
<td>EST</td>
<td>AH427061</td>
<td>AT7</td>
<td>17</td>
<td>5</td>
</tr>
<tr>
<td>Gp250 precursor/sortilin-related</td>
<td>AF031816</td>
<td>AT7</td>
<td>9</td>
<td>11q23.2–q24.2</td>
</tr>
<tr>
<td>Mouse four cell embryo cDNA clone</td>
<td>AU042200</td>
<td>AT7</td>
<td>16</td>
<td>17</td>
</tr>
<tr>
<td>Mouse TNF-receptor 2</td>
<td>M60469</td>
<td>AT7</td>
<td>4; 75.5 cm</td>
<td>1p36.3–p36.2</td>
</tr>
<tr>
<td>Mouse valosin containing protein</td>
<td>NM_009503</td>
<td>AT7</td>
<td>4; 23.0 cm</td>
<td>9p13–p12</td>
</tr>
<tr>
<td>Absent in melanoma (AIM2) Human (confirmed)</td>
<td>AF024714</td>
<td>AT7</td>
<td>1</td>
<td>1q22</td>
</tr>
<tr>
<td>Interleukin 4 receptor, alpha (confirmed)</td>
<td>NM_010557</td>
<td>AT7</td>
<td>7; 62.0 cm</td>
<td>16p11.2–12.1</td>
</tr>
<tr>
<td>Unknown clone</td>
<td>Y17677</td>
<td>AT7</td>
<td>1</td>
<td>16</td>
</tr>
<tr>
<td>Mouse ALK-1 (confirmed)</td>
<td>Z31664</td>
<td>AT12</td>
<td>15</td>
<td>12q11–q13</td>
</tr>
<tr>
<td>Mouse rah g-protein</td>
<td>S72304</td>
<td>AT12</td>
<td>11; 44.8 cm</td>
<td>17</td>
</tr>
<tr>
<td>EST; novel (confirmed)</td>
<td>AB041555</td>
<td>AT12</td>
<td>4</td>
<td>17</td>
</tr>
<tr>
<td>EST: S31814 ADP, ATP carrier protein T2</td>
<td>AJ854173</td>
<td>AT12</td>
<td>11</td>
<td>17</td>
</tr>
<tr>
<td>Annexin XI (Anx11)</td>
<td>U65986</td>
<td>AT12</td>
<td>14; 3.3</td>
<td>10q22–q23</td>
</tr>
<tr>
<td>28S ribosomal RNA</td>
<td>X00525</td>
<td>AT12</td>
<td>N/a</td>
<td>n/a</td>
</tr>
<tr>
<td>EST</td>
<td>BF320258</td>
<td>AT12</td>
<td>17</td>
<td>6</td>
</tr>
<tr>
<td>Rat RSTK-1 (confirmed)</td>
<td>L36088</td>
<td>AT12</td>
<td>15</td>
<td>12q11–q14</td>
</tr>
<tr>
<td>Soluble lectin (Mac-2) gene (confirmed)</td>
<td>LO8649</td>
<td>AT13</td>
<td>14</td>
<td>14q21–q22</td>
</tr>
<tr>
<td><strong>Genes with decreased expression in a single tumor cell line</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-4 receptor secreted form</td>
<td>M27960</td>
<td>AT4</td>
<td>7; 62.0 cm</td>
<td>16p11.2–12.1</td>
</tr>
<tr>
<td>FX-induced thymoma transcript (confirmed)</td>
<td>U38252</td>
<td>AT4</td>
<td>5; 65.0 cm</td>
<td>12</td>
</tr>
<tr>
<td>Mouse p162/centrosin/EIF3</td>
<td>X84651</td>
<td>AT7</td>
<td>19</td>
<td>10q26</td>
</tr>
<tr>
<td>EST; novel</td>
<td>AA259694</td>
<td>AT7</td>
<td>7</td>
<td>19</td>
</tr>
<tr>
<td>Novel</td>
<td>AV065690</td>
<td>AT7</td>
<td>11</td>
<td>17</td>
</tr>
<tr>
<td>Major histocompatibility complex Q region</td>
<td>AF111103</td>
<td>AT7</td>
<td>17</td>
<td>n/a</td>
</tr>
<tr>
<td>CTP synthetase</td>
<td>U49350</td>
<td>AT7</td>
<td>4; 57.0 cm</td>
<td>1p34.1</td>
</tr>
<tr>
<td>ADP-ribosylation factor (confirmed)</td>
<td>NM_007476</td>
<td>AT7</td>
<td>1</td>
<td>1q42</td>
</tr>
<tr>
<td>Homology to human CGI-94</td>
<td>AF151852</td>
<td>AT12</td>
<td>4</td>
<td>1q42</td>
</tr>
<tr>
<td>Mouse mitochondrial DNA</td>
<td>AB049357</td>
<td>AT12</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>T-cell receptor rearranged gamma chain</td>
<td>M34970</td>
<td>AT12</td>
<td>13; 10.0 cm</td>
<td>7p15–p14</td>
</tr>
<tr>
<td>Rat Histone macroH2A.2</td>
<td>U79139</td>
<td>AT12</td>
<td>13</td>
<td>5q31.3–q32</td>
</tr>
<tr>
<td>Chimeric 16S ribosomal RNA (mito)</td>
<td>AF089815</td>
<td>AT12</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>K-ras type A mRNA, 3' untranslated</td>
<td>U76425</td>
<td>AT12</td>
<td>6; 71.2 cm</td>
<td>12p12.1</td>
</tr>
<tr>
<td>COQ7</td>
<td>AF098949</td>
<td>AT12</td>
<td>7; 53.5 cm</td>
<td>16p13.11–p12.3</td>
</tr>
<tr>
<td>Prothymosin alpha</td>
<td>NM_008972</td>
<td>AT12</td>
<td>[1]</td>
<td>2q35–q36</td>
</tr>
<tr>
<td>Mini chromosome maintenance deficient 7</td>
<td>NM_008568</td>
<td>AT12</td>
<td>[16]</td>
<td>7q21–q22.1</td>
</tr>
<tr>
<td>EST from embryonic carcinoma</td>
<td>AA213215</td>
<td>AT12</td>
<td>1</td>
<td>1q21</td>
</tr>
<tr>
<td>EST from thymus</td>
<td>BE631434</td>
<td>AT12</td>
<td>1</td>
<td>n/a</td>
</tr>
<tr>
<td>Homology to human YG81 hypothetical protein</td>
<td>XM_009703</td>
<td>AT12</td>
<td>16</td>
<td>21q21.1</td>
</tr>
<tr>
<td>Inhibitor of Apoptosis 1 (confirmed)</td>
<td>U88908</td>
<td>AT12</td>
<td>9 A2</td>
<td>11q22</td>
</tr>
<tr>
<td>Homology to rat Pxm1</td>
<td>NM_012804</td>
<td>AT13</td>
<td>3; 56.6 cm</td>
<td>1p22–p21</td>
</tr>
<tr>
<td>Homology to rat glucokinase</td>
<td>AF217233</td>
<td>AT13</td>
<td>1</td>
<td>1q21–q22</td>
</tr>
<tr>
<td>EST; Novel (confirmed)</td>
<td>AA719318</td>
<td>AT13</td>
<td>11</td>
<td>2q21</td>
</tr>
<tr>
<td>COP9 subunit 4</td>
<td>NM_012001</td>
<td>AT13</td>
<td>5</td>
<td>4q21.21–q21.23</td>
</tr>
<tr>
<td>EST</td>
<td>AW824167</td>
<td>AT13</td>
<td>11</td>
<td>17</td>
</tr>
<tr>
<td>Rat histone macroH2A1.2 mRNA</td>
<td>U79139</td>
<td>AT13</td>
<td>13</td>
<td>5q31.3–q32</td>
</tr>
<tr>
<td>Kidney testosterone-regulated RP2</td>
<td>X04097</td>
<td>AT13</td>
<td>7; 15.0 cm</td>
<td>[19]</td>
</tr>
<tr>
<td>Replication dependent histone H2A.1</td>
<td>M37736</td>
<td>AT13</td>
<td>13</td>
<td>1q21</td>
</tr>
<tr>
<td>EST to NMLMG cDNA clone (confirmed)</td>
<td>AI461777</td>
<td>AT12</td>
<td>11</td>
<td>5</td>
</tr>
<tr>
<td><strong>Genes with increased or decreased expression in two of four tumor cell lines</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mouse mo54 protein</td>
<td>J05261</td>
<td>AT4–AT12</td>
<td>2; 96.0 cm</td>
<td>20q13.1</td>
</tr>
<tr>
<td>Rat ketohexokinase promoter region</td>
<td>Y09339</td>
<td>AT4–AT12</td>
<td>5; 18.1 cm</td>
<td>2p23.2–p23.3</td>
</tr>
<tr>
<td>Unknown clone from E16 pancreas library</td>
<td>BG142044</td>
<td>AT4–AT12</td>
<td>3</td>
<td>1</td>
</tr>
</tbody>
</table>

Continued
found at the chromosome 12/14 breakpoint in AT-7, a similar translocation of the adjacent variable region of the TCRα locus to chromosome 12 was not observed (Fig. 3C, upper panel).

To determine whether the rearrangement and an aberrant transcript could be detected in multiple independent Atm−/− TCLs or in p53−/− TCLs, rapid amplification of cDNA ends (RACE) was performed using RNA from the original TOGA® samples, as well as from other Atm−/− and p53−/− TCLs. Using 5’ RACE and RNA derived from AT-7 and AT-10 (not used in the TOGA® analysis), the full-length coding sequence was obtained. Sequencing of the clones showed that the aberrant message produced a transcript encoding an identical in-frame fusion between Gzm B and C (see Fig. 3D). Subsequent RT-PCR analysis using primers specific for GzmB and GzmC confirmed the presence of a similar abnormal fusion between Gzm B and C in the AT-7, AT-10 and AT-13 cell lines, and demonstrated other aberrant rearrangements in several other Atm−/− cell lines. In total, five of the six Atm−/− TCLs examined showed abnormal products derived from the granzyme locus. In contrast, no such transcript was found in RNA from normal mouse thymus or from p53−/− TCLs, confirming that aberrant GzmC products are only seen in Atm−/− TCLs. Interestingly, a core 20 bp sequence [TGC(T/A)(A/G)TGTTGCTGTGGGG] is found in all six granzymes residing on mouse chromosome 14, and is less conserved in mouse GzmA and GzmK (both on chromosome 13), and GzmM (chromosome 10). This core site is conserved in the GzmB–C fusions arising in independent Atm−/− TCLs (Fig. 3D). Therefore, not only was the identical GzmB–C fusion observed in independent Atm−/− TCLs, but also the site of the fusion occurs within a highly conserved region between the granzyme family members on chromosome 14.

Importantly, the aberrant in-frame fusion transcript between GzmB and GzmC in Atm−/− TCLs retains all functional domains (62–69). To determine whether the transcript detected in the Atm−/− TCLs was capable of generating an intact protein, the GzmB–C fusion sequence was His-tagged using the arabinose-inducible vector pBAD-HisG (Invitrogen). Induction of this construct resulted in the production of a protein of the expected size (25.8 kDa), as determined by western blot analysis with an anti-His antibody (Fig. 3E). Taken together, these results demonstrate that the rearrangements of the granzyme locus are found only in the absence of Atm and also that the sites of fusion involve a region of sequence homology between the family members and that the aberrant product with unique properties contains all functional domains necessary for activity.

**DISCUSSION**

**Lymphomagenesis in the absence of ATM**

The results we have obtained enabled us to develop a model for lymphomagenesis arising in the absence of functional ATM (Fig. 4). Loss of ATM results in the destruction of most CD4+ /CD8+ T-cells. It is thought that this defect in T-cell maturation is due to the compromised ability of Atm−/− T-cells to appropriately produce a functional TCR (see Fig. 4). Those CD4+/CD8+ T-cells that do not appropriately rearrange TCRα and express a TCR undergo apoptosis (see Fig. 4) (27). Importantly, in the absence of ATM many T-cells do undergo productive TCRα/β rearrangement and mature to become functional T-cells, although we and others have demonstrated that many of these ‘functional’ T-cells harbor chromosomal abnormalities. These abnormalities neither appear to affect the function of the cells nor give rise to cells that eventually cause lymphoma leukemia as indicated by the fact that the Atm−/− tumors are not CD3+.

Our profiling experiments suggest that cancerous cells must arise in the less mature CD4+/CD8+ cells residing in the thymus (Fig. 4). Gene expression profiling showed that tumor-free Atm−/− thymus abnormally expressed several genes, consistent with the idea that a subset of abnormal cells harbors changes important for the pre-cancerous phenotype (Fig. 2A). The TCRγ locus is frequently abnormally rearranged in non-tumorigenic peripheral T-cells in A-T patients. Normally, during the process of TCRα/β maturation, expression of TCRγ is downregulated regardless of the presence of a productive TCRγ rearrangement (70). It is therefore possible that persistent expression of TCRγ in the absence of ATM is due to the lack of a productively rearranged TCRα or TCRδ allele in the setting of a productive TCRγ rearrangement in these pre-cancerous cells. These cells are able to survive when arrested at the CD4+/CD8+ stage. This type of cell must be prone to becoming cancerous, because TCRγ expression was abnormal in both the Atm−/− thymus and
the \( Atm^{+/−} \) TCLs. In further support of this hypothesis is the finding that in the more mature \( p53^{+/−} \) TCLs, TCR\(\alpha\) rearrangement is not compromised, there is no similar abnor-
mal expression of TCR\(\gamma\).

Cystatin C is misregulated in pre-cancerous \( Atm^{+/−} \) thymus and in both the \( p53^{+/−} \) and \( Atm^{+/−} \) TCLs, and others have shown that cystatin C expression is altered in other T-cell cancers (44,45,71,72). It is possible that decreased expression of cystatin C provides a survival advantage for abnormal T-cells in the \( Atm^{+/−} \) thymus and later as tumors develop may increase their invasive capacity.

Common and unique gene expression changes in various T-cell-derived cancers

How then do pre-malignant \( Atm^{+/−} \) thymocytes become cancerous? One possibility is that continued attempts to produce a viable TCR result in genomic instability that leads to a series of specific lesions. These disruptions (assumed to involve the TCR or Ig genes or regulatory elements) could either activate or inactivate key oncogenes or tumor suppressor genes. Although V(D)J recombination is impaired in the absence of ATM, it is not abolished, as some mature T-cells are present.

Figure 3. Chromosomal rearrangement in \( Atm^{−/−} \) TCLs results in generation of an aberrant fusion within the Gzm gene cluster. (A) Ideogram and cytogenetic map of mouse chromosome 14 showing the locations of the Gzm family gene cluster spanning 20.5–21 cm and the TCR\(\alpha\) locus at 19.5–19.7 cm. (B) Northern blot showing normal (*) and aberrant (**) transcripts of GzmC. Aberrant transcripts were observed in three of five \( Atm^{−/−} \) TCLs (lanes 2, 3 and 6) and one \( Atm^{+/−} p53^{+/−} \) TCL (APT-3, lane 7) but not in \( Atm^{−/−} p53^{−/−} \) TCLs (lanes 9 and 10), \( p53^{−/−} \) TCL (lane 8) or normal thymus (lane 11). Methylene blue staining in the lower panel shows equal loading of samples. (C) FISH of \( Atm^{−/−} \) TCL (AT-7) metaphase chromosomes hybridized with probes for GzmC (green, chromosome 14), TCR\(\alpha\) variable region (red, chromosome 14) and Tcl1 (red, chromosome 12). Duplication and inversion of the GzmC locus on chromosome 14 is observed, as well as a translocation of the GzmC locus to chromosome 12 near the Tcl1 locus. Translocation of the TCR\(\alpha\) variable locus to chromosome 12 is not observed. (D) The sequence and structure of the aberrant Gzm transcript from multiple \( Atm^{−/−} \) TCLs is shown. The site of the fusion corresponding to the third exon of GzmB with third exon of GzmC is indicated. This is a site of overlapping homology of 23 bp (shown in black) between the two Gzm genes. The lower panel indicates where the junction occurs in the fusion sequence. (E) Western blot using an anti-His antibody following arabinose induction of the His-tagged GzmB–C fusion shows that a protein of expected molecular weight (25.8 kDa indicated by an arrowhead) is produced. Lanes 1–5 show protein extracts induced with increasing arabinose concentrations (0, 0.002, 0.02, 0.2 and 2%). Expression of a control His-tagged LacZ construct (LacZ) is shown in lanes 7 (uninduced) and 8 (2% arabinose).
it is possible that the misregulation of these and other genes promote the survival of the abnormal TCR expressing cells that should have been cleared by apoptosis, although their aberrant expression alone is not sufficient to give rise to a cancer cell. Subsequently, continued genomic instability results in the rearrangements/disruptions of loci on chromosome 12. Aberrations of the TCR family may result in the expression of an aberrant product that imparts the proliferative and invasive properties of these tumors.

in the periphery of both Atm−/− mice and A-T patients. In addition, no clear defects have been found in V(D)J recombination involving the TCRβ or Ig loci. In fact, B-cell function and numbers are virtually normal in the majority of patients and in Atm−/− mice (3,6,24). Most lymphomas/leukemias found in A-T patients are of T-cell origin. Only rarely do A-T patients develop B-cell lymphomas, and B-cell lymphomas are only found in mice with combined deficiencies in ATM and other genes, such as p53 (6) (and data not shown). In addition, DNA repair kinetics are normal in A-T (73,74). However, in both Rag1/ATM- and Rag2/ATM-deficient mice, V(D)J recombination is prevented, yet mice still succumb to TCLs (even much less rapidly) and these TCLs do not harbor abnormalities at the TCRα locus. Taken together, these data suggest it is unlikely that abnormalities in V(D)J recombination alone are responsible for tumor formation that a lesion on chromosome 14 near the TCRα locus is essential for the rapid onset of aggressive lymphomas in Atm−/− thymocytes.

The successful combination of immunophenotyping, clinical characterization, cytogenetic analysis and RNA profiling techniques led us to a better understanding of the genes affected by the conserved aberrations in Atm−/− TCLs and identified the granzyme gene cluster on chromosome 14 as consistently abnormal. This is the first demonstration of a translocation event that results in the production of an in-frame fusion between granzyme family members that yields a coding sequence for an intact protein. The distance between the fusion sites is 27 kb, and so it is unlikely that the increased expression is due to aberrant splicing events. Importantly, we show that the GzmB−C fusion observed in multiple Atm−/− TCLs is identical and the site of fusion maintains all the regions necessary for full activity (62,69).

The three catalytic residues come from the fusion between GzmB and GzmC and the active site serine is derived from GzmC (62,75,76). Increased GzmB expression has already been reported in many T-cell tumors with poor clinical outcomes (64,65,68,77) where increased expression is thought to be an adoptive mechanism that enables tumors to actively destroy host immune effector cells and invade tissues (63,66,78–80). However, prior to this study, the expression of granzymes in tumors has not been associated with aberrations involving the locus. Clearly it will be interesting to test the effect of the observed granzyme fusion on normal and transformed cells in future studies to better define a role for this chromosomal aberration in tumors.

Finally, the disruption of a specific locus on chromosome 12 appears to be essential for tumor formation in the absence of ATM (see Figs 3 and 4). This locus may be of critical significance, as all Atm−/− and Rag−/− Atm−/− tumor studies to date have aberrations on chromosome 12D-F. We know that the lesion involves regions near (but not affecting) the Tcl1 locus and only rarely the IgH locus (4,6), although the genes and loci involved remain to be determined.

Figure 4. Mechanism of progression from thymocyte to TCL in the absence of ATM. In the absence of ATM, T-cells undergo rearrangement of TCRβ and progress to the CD4+/CD8− stage. At this stage, the T-cell population undergoes rapid expansion and begins to rearrange the TCRα locus. Thymic selection of T-cells with non-functional TCR rearrangements occurs at this stage. In the absence of ATM, a small number of Atm−/− T-cells undergo proper rearrangement and migrate as functional T-cells to the periphery. These cells have single disruptions of chromosomes 12 or 14 in mice and 7 and 14 in human. However, many of these CD4+/CD8− cells are unable to express a functional TCR. The majority of these cells undergo apoptosis, but a few escape thymic selection, and these pre-malignant cells are arrested at the CD4+/CD8− stage of maturation. These cells likely harbor single disruptions of chromosome 12 or 14 and show misregulation of a few genes. A small number of genes show persistent misregulation in the cancer cell population, including TCRγ, Igα, cystatin C and an EST. These data suggest it is unlikely that abnormalities of granzymes in tumors has not been associated with aberrations involving the locus. Clearly it will be interesting to test the effect of the observed granzyme fusion on normal and transformed cells in future studies to better define a role for this chromosomal aberration in tumors.
Aberrant HR as a consequence of dysfunctional NHEJ leads to tumorigenesis in Atm−/− TCLs

In A-T patients, no type of cell is consistently free of increased chromosomal breakage. Similar genetic instability is observed in Bloom’s syndrome, which shares many of the same phenotypic characteristics of A-T including immunodeficiency, growth retardation and predisposition to cancers (81,82). Interestingly, the genetic instability in Bloom’s syndrome results from increased HR and an elevated level of somatic mutations. Furthermore, the Bloom protein interacts directly with ATM and undergoes phosphorylation by ATM in response to IR (83). These observations indicate that ATM may be involved in regulating HR in response to DNA damage. Our results demonstrating specific disruption of genes in the Gzm cluster were particularly surprising. The observation that the granzyme gene family cluster, near the TCRα/δ locus on chromosome 14, is disrupted in the development of ATM-deficient lymphomas suggests a role for compensatory repair pathways to assist with the impaired nature of these tumors and point to potential therapeutic choices.

Human Molecular Genetics, 2005, Vol. 14, No. 18

Flow cytometry for phenotyping of tumors
Flow cytometry and phenotyping of tumors were done as described (3).

SKY and FISH
Metaphase spreads for SKY and FISH were prepared on glass slides using standard protocols as described in Ref. (86). Cells were incubated in 0.1 mg/ml Colcemid (GIBCO/BRL) for 30–60 min and then lysed in 0.075 M KCl. Chromosomes were fixed in 3:1 methanol:acetic acid and dropped onto glass slides. SKY was performed as described (87,88). Six to ten metaphases were analyzed for each tumor. Probes for FISH were generated using bacterial artificial chromosome (BAC) clones containing the genes of interest. BAC clones were obtained by PCR screening of Down-to-the-Well pools according to the manufacturer’s protocol (Genome Systems, St. Louis, MO). The clone addresses for the isolated BAC clones were as follows: 226E11 (Tcl1), 232F19 (Tcrα), 46G9 (TcrVo6), and 309K16 and 380N13 (GzmC). Labeled BAC probes were generated using the BioProbe nick-translation kit (Sigma). The BAC DNA clones were labeled with biotin-16-dUTP, digoxigenin-11-dUTP (Roche), or Spectrum Orange-dUTP (Vysis, Downer’s Grove, IL). Hundred nanograms of nick-translated probe DNA was precipitated with 15 μg Mouse C57-1 DNA (Gibco) and resuspended in 50% formamide, 10% dextran sulfate, 2× SSC. The probe DNA was denatured (10 min at 75°C) and metaphase spreads were pretreated with RNase A (0.1 mg/ml, for 1 h at 37°C) and pepsin (0.1 mg/ml for 10 min at 37°C) followed by fixation in formalin (1%, for 10 min at room temperature). After 30-min preannealing of probe DNA, hybridization to metaphase spreads was carried out for 24 h, at 37°C in a humidified box as previously described (89). After hybridization, indirectly labeled probes were detected by either mouse antidigoxigenin followed by sheep anti-mouse Cy5.5, or avidin FITC. FISH results were imaged and analyzed using QFISH software (Leica, Cambridge, UK).

Isolation of tissue and RNA
Thymus from age- and sex-matched pairs of wild-type and Atm−/− 129S6/SvEvTAC inbred mice were dissected between 4 and 16 weeks of age. Thymus was visually inspected for tumor foci and tumor-free samples were flash-frozen on dry ice and stored at −80°C until used for RNA isolation. RNA was isolated from TCLs and was used for TOGA® as described (47). RNA used in northern blotting and microarray analysis was isolated using TRIZol Reagent for thymus or TRIZol LS Reagent (Gibco-BRL) for cell lines. RNA quality was assessed by spectrophotometry and gel electrophoresis; RNA with A260/A280 ratios greater than 2.0 in TE and no visible evidence of degradation by electrophoresis was used for northern blot analysis and expression profiling.

MATERIALS AND METHODS

Cell culture
Tumor cell lines were isolated as previously described (3) and grown in RPMI medium (Life Technologies, Bethesda, MD) with 10% heat-inactivated fetal calf serum and 20 U/ml human interleukin-2 (Roche).
Identification of differentially expressed transcripts

TOGA® was carried out on duplicate samples of four independently isolated and characterized cells lines at passages 3 to 5. Initial candidate selections were made with the TOGA® portal using an in-house algorithm for peak detection and analysis to discriminate fold changes across samples after normalization (49). Following selection of initial candidates with the TOGA® portal, trace patterns were examined by eye, and distinct peaks with expression levels greater than 100 relative fluorescence units were selected. Sixty-five candidates were selected for follow-up analysis. The clones representing the 3′ regions of all 65 candidates identified by TOGA® were obtained, their sequences determined and compared with the GenBank database.

Northern blotting

Ten micrograms of total RNA per lane was used for northern blot analysis following the glyoxal denaturation protocol (90). Gels were transfer blotted onto HybondTM N membrane (Amersham Pharmacia), washed and crosslinked following standard procedures (91). Blots were stained for RNA loading with 0.5 M acetic acid (pH 5.2), 0.04% methylene blue and destained in 0.1 M acetic acid (pH 5.2), 0.04% methylene blue and destained in ddH2O. Gels corresponding to mouse GzmC (AA389537) were obtained (Genome Systems, Inc. and Digital Gene Technologies) and sequence verified. Fragments were gel purified using the QIAquick® Gel Extraction Kit (Qiagen) and 32P random prime labeled using the RediprimeII labeling system (Amer sham). Fragments for mouse β-actin, cyclophilin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were obtained commercially (Ambion) and labeled as described above. Labeled probes were hybridized at a specific activity of 1 × 106 cpm/ml in Church’s Buffer following standard procedures (92). Blots were visualized on PhosphorImager screens overnight and signals quantified using ImageQuant software (Molecular Dynamics).

TaqMan quantitative RT-PCR analysis

Primers for TaqMan Quantitative RT-PCR analysis were designed using Primer Express 1.0 software (PE Biosystems). The cDNA used in the PCR analysis was synthesized from total RNA using Superscript II Reverse Transcriptase (Invitrogen). The primers for TCRγ amplified an 84-bp fragment and were forward primer (5′-CAGGAGGGTGCTGTAGCTGCT-3′) reverse primer (5′-GCTTTTGTCAGAGGAAATTAGTATG-3′). The CD53 primers amplified a 78-bp fragment and were forward primer (5′-ACCATCTCTTCTGCCCATCAG-3′) and reverse primer (5′-TGCGATGTCTGCGGTGTATGATAAGGCCCA-3′). Results were normalized using β-actin to amplify a 69-bp fragment using forward primer (5′-GGGCCTTTTGACCTGAGGAT-3′) and reverse primer (5′-GGATTTGTGCTCACAACCA-3′).

5′ RACE

Analysis of Gzm fusions was carried out by RNA ligase-mediated rapid amplification of cDNA ends (RLM-RACE), using the GeneRacer Kit (Invitrogen). Experiments were performed according to the protocol using oligo-dT primers to generate RACE-ready cDNA. DNaseI-treated RNA samples used to generate the original TOGA® libraries (AT-3, AT-4, AT-7, AT-12 and AT-13) were used as templates for RACE. In addition, RNAs from Atm+/− (AT-10) and Atm−/− p53+/− (APT-3) TCLs and from wild-type thymus were analyzed.

Microarray experiments and data analysis

cDNA microarrays were prepared at the Salk Institute Functional Genomics Laboratory using 9216 sequence-confirmed mouse Unigene cluster cDNAs obtained from Genome Systems (Palo Alto, CA). Clones were spotted on amino-silane-coated, aluminized glass slides in duplicate. Total RNA for hybridization to cDNA microarrays was either labeled directly using aminomethyl labeling or was amplified using a single round in vitro transcription (IVT) reaction and then labeled using aminoalkyl labeling. Aminoalkyl labeling using 10 μg of total RNA was performed essentially as described (http://cmgm.stanford.edu/pbrown/protocols/aadUTP CouplingProcedure.htm).

IVT amplification was performed using 2 μg of total RNA and reverse transcription (RT) with a T7-d(T)24 primer (Geneset). cDNA was extracted from the RT reaction, and purified using a Microcon C50 spin column (Millipore). Single round amplification of purified cDNA was performed using the MAXiScriptTM IVT kit (Ambion), and complementary RNA (cRNA) purified using the RNeasy® Mini Kit (Qiagen). Three micrograms of IVT cRNA was labeled using the aminoalkyl protocol described above. Cy5- and Cy3-labeled cDNAs were quantified by spectrophotometry. Twenty picomoles of labeled Cy5 sample and 20 pmol of Cy3 sample were lyophilized for use in the hybridization.

Pretreated slides were hybridized with 40 μl hybridization solution (20 μl formamide, 10 μl 4× Hybridization buffer v.2 (Amersham), 5 μg mouse C0,1 DNA (Invitrogen), 5 μg polyadenylic acid (Sigma), 20 pmol of the labeled Cy5 and 20 pmol of the labeled Cy3 DNA samples in the dark for 16 h at 42°C in humidified CMTTM Hybridization Chambers (Corning). After hybridization, slides were washed, dried with compressed air and scanned immediately (Molecular Dynamics Array Scanner GenIII). Additional information about the cDNA microarray protocols and data analysis methods are provided as supplemental data.

Database analyses

Public databases searched in these analyses were NCBI (http://www.ncbi.nlm.nih.gov) (including LocusLink, GenBank, Mouse–Human Homology Maps and Unigene) and the Mouse Genome Database (http://www.informatics.jax.org) (93). In addition, data were generated through use of the Celera Discovery System and Celera’s associated databases (94).
ACKNOWLEDGEMENTS

The authors thank Daniel J. Lockhart and Matthew Zapala for the development of the SPOT software for microarray data analysis, David Lo and Julie Ellison for help with TOGA data analysis and support, Ling Ouyang, Joel Lachuer and the Salk Functional Genomics Laboratory for assistance with microarray experiments, Rob Helton for animal husbandry, Jamie Simon for artwork and Danny Wangsa for assistance with cytogenetic analyses. The Salk Functional Genomics Laboratory is supported by the H.N. and Frances C. Berger Foundation, the Lebensfeld Foundation, Charles R. Pollock, the Scher Family Foundation, Inc., the Salk Institute Association and an NIH Cancer Core Grant (W. Eckhart). C.W. is supported by the Canadian Institutes of Health Research. D.P. is supported by the Chapman Charitable Trust. C. B. is supported by the Scher Family Foundation, Inc., the Salk Institute and the Salk Functional Genomics Laboratory for assistance with data analysis and support, Ling Ouyang, Joel Lachuer and analysis, David Lo and Julie Ellison for help with TOGA the development of the SPOT software for microarray data analysis, and T-cell prolymphocytic leukemia.

REFERENCES

78. Ronday, H.K., van der Laan, W.H., Tak, P.P., de Roos, J.A., Bank, R.A.,
TeKoppele, J.M., Froelich, C.J., Hack, C.E., Hogendoorn, P.C.,
Breedveld, F.C. et al. (2001) Human granzyme B mediates cartilage
proteoglycan degradation and is expressed at the invasive front of the

79. Froelich, C.J., Zhang, X., Turbov, J., Hudig, D., Winkler, U. and

(2002) Expression of the granzyme B inhibitor, protease inhibitor 9, by
protective mechanism for tumor cells to circumvent the immune

81. Mohaghegh, P., Karow, J.K., Brosh, R.M., Jr, Bohr, V.A. and
Hickson, I.D. (2001) The Bloom’s and Werner’s syndrome proteins are


83. Beamish, H., Kedar, P., Kaneko, H., Chen, P., Fukao, T., Peng, C.,
Functional link between BLM defective in Bloom’s syndrome and the
ataxia-telangiectasia-mutated protein, ATM. *J. Biol. Chem.*, 277,
30515–30523.

Atm deficiency causes an increased frequency of intrachromosomal

85. Motyka, B., Korbutt, G., Pinkoski, M.J., Heibein, J.A., Caputo, A.,
Holman, M., Bary, M., Shostak, I., Sawchuk, T., Holmes, C.F. et al.
(2000) Mannose 6-phosphate/insulin-like growth factor II receptor is a
death receptor for granzyme B during cytotoxic T cell-induced apoptosis.
*Cell*, 103, 491–500.

86. Henegariu, O., Heerema, N.A., Lowe Wright, L., Bray-Ward, P.,
preparation: controlled chromosome spreading, chemical aging and

87. Schrock, E., du Manoir, S., Veldman, T., Schoell, B., Wienberg, J.,
Ferguson-Smith, M.A., Ning, Y., Ledbetter, D.H., Bar-Am, I.,
Soenksen, D. et al. (1996) Multicolor spectral karyotyping of human

88. Liyanage, M., Coleman, A., du Manoir, S., Veldman, T., McCormack, S.,
Dickson, R.B., Barlow, C., Wynshaw-Boris, A., Janz, S., Wienberg, J.

89. Kerlavage, A., Bonazzi, V., di Tommaso, M., Lawrence, C., Li, P.,

non-radioactive northern blot analysis of nerve growth factor mRNA from

91. Blake, J.A., Eppig, J.T., Richardson, J.E., Bult, C.J. and Kadin, J.A.
(2001) The Mouse Genome Database (MGD): integration nexus for the

92. Kerlavage, A., Bonazzi, V., di Tommaso, M., Lawrence, C., Li, P.,


Cloning—A Laboratory Manual*, 2nd edn. CSH Laboratory Press, Cold
Spring Harbor.

non-radioactive northern blot analysis of nerve growth factor mRNA from

96. Blake, J.A., Eppig, J.T., Richardson, J.E., Bult, C.J. and Kadin, J.A.
(2001) The Mouse Genome Database (MGD): integration nexus for the