EPHA7, a new target gene for 6q deletion in T-cell lymphoblastic lymphomas

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Cryptic deletions at chromosome 6q are common cyogenetic abnormalities in T-cell lymphoblastic leukemia/lymphoma (T-LBL), but the target genes have not been formally identified. Our results build on detection of specific chromosomal losses in a mouse model of γ-radiation-induced T-LBLs and provide interesting clues for new putative susceptibility genes in a region orthologous to human 6q15–6q16.3. Among these, Epha7 emerges as a bona fide candidate tumor suppressor gene because it is inactivated in practically all the T-LBLs analyzed (100% in mouse and 95.23% in human). We provide evidence showing that Epha7 downregulation may occur, at least in part, by loss of heterozygosity (19.35% in mouse and 12.25% in human) or promoter hypermethylation (51.61% in mouse and 43.75% in human) or a combination of both mechanisms (12.90% in mouse and 6.25% in human). These results indicate that EPHA7 might be considered a new tumor suppressor gene for 6q deletions in T-LBLs. Notably, this gene is located in 6q16.1 proximal to GRIK2 and CASP8AP2, other candidate genes identified in this region. Thus, del6q seems to be a complex region where inactivation of multiple genes may cooperatively contribute to the onset of T-cell lymphomas.

Introduction

T-cell acute lymphoblastic leukemia (T-ALL) represents 15 and 25% of all newly diagnosed cases of acute lymphoblastic leukemia in children and adults, respectively. Despite the ever-increasing understanding of the molecular pathogenesis in recent years, essential data about the genetic features underlying the pathophysiology of these malignancies remain to be elucidated (1). A recurrent cytogenetic abnormality in T-ALL is del6q of variable size, which occurs in 20–30% of cases (2). In this region, GRIK2 has been identified as the gene most frequently affected by 6q16 deletions in T-ALL (3). However, other authors have reported CASP8AP2 as the most downregulated gene in the deleted region at 6q15–16.1 (4). In view of this complexity, it is reasonable to think that not all genes involved in 6q deletions have yet been formally identified (2).

In addition to T-cell leukemia/lymphoma, recurrent deletions at 6q have also been described in multiple kinds of cancers, including B-cell lymphomas (5,6), multiple myeloma (7), laryngeal squamous cell carcinoma (8), non-hereditary non-polyposis colorectal cancer and non-familial adenomatous polyposis-associated intestinal carcinomas (9), uveal melanoma (10), pediatric intracranial ependymomas (11), ovarian carcinoma (12), radiation-induced papillary thyroid carcinomas (13), breast cancer (14), etc., highlighting the importance of this region in cancer development.

Given the broad similarities between T-ALL and T-cell lymphoblastic lymphomas (T-LBLs), the distinction between these two entities is now understood to be artificial by most oncologists (15). Thus, we hypothesized that 6q deletion and/or inactivation of target genes in this region may likewise be present in T-LBLs. In addition, the use of mouse models would be a valuable strategy to facilitate the identification of target susceptibility genes in this deletion site.

Ionizing γ-radiation is known to induce double-strand breaks that lead to translocations, deletions and amplifications; thus, we reasoned that the classical model of γ-radiation-induced T-LBLs in mouse (16–18) could be an excellent tool to reproduce chromosomal deletions that would be orthogonal to those described in humans. Using this model, frequent loss of heterozygosity (LOH) has been reported in a region on chromosome 4 (thymus lymphoma suppressor region 5, TLSR5) that is orthologous to the human 6q16 (http://www.ensembl.org/Homo-sapiens/Locus/Synteny) (19). In this study, we define more precisely the boundaries of LOH regions detected on mouse chromosome 4 using new molecular markers and an extended collection of γ-radiation-induced T-LBL samples. Our results provide the first evidence of inactivation of several genes in this region, which is syntenic with human chromosome 6q16, Epha7 being the most severely affected one.

Materials and methods

Mice colony and T-cell lymphoma induction

C57BL/6J and BALB/cJ mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Animal experiments were carried out according to the European Commission Guidelines (Directive 86/609/CEE) on the use of laboratory animals. For T-cell lymphoma induction, 4-week-old female mice were exposed to whole-body fractionated (4 × 1.75 Gy) gamma irradiation at weekly doses, following the protocol approved by our Committee of Bioethics and Animal Care (Ethic Committee of Autonomous University of Madrid, CEI 22-478). Treated mice were observed at weekly intervals beginning 12 weeks after completion of γ-irradiation treatment and up to 25 weeks (the latency period for these tumors) and phenotyped for the presence/absence of thymic lymphomas as we described previously (16). Mouse sample characteristics are shown in Supplementary Table S1, available at Carcinogenesis online.

Human samples

Thymus of human fetus, without pathology, and human T-LBLs were obtained from the Spanish Tumour Bank Network of the Spanish National Cancer Research Centre (Centro Nacional de Investigaciones Oncológicas, CNIO). Institutional review board approval was obtained for these studies (reference RNDdT 10/073), and the participants provided written informed consent in accordance with the Declaration of Helsinki. Additional information is provided as Supplementary Table S1, available at Carcinogenesis online.

Cell line and treatment

Jurkat cell line derived from T-ALL/T-LBLs was obtained from the German National Tissue Cultured Collection (DSMZ) and grown using standard procedures. Jurkat cells were treated with 5 μM 5-aza-2 deoxycytidine for 72 h in order to assess the involvement of epigenetic effects on EPHA7 expression.

Isolation and purification of DNA and RNA

DNA from mouse and human samples were extracted using standard phenol–chloroform method. Total RNA was isolated from cells and tissues with Trizol (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions.

LOH analysis

LOH analysis was carried out by comparing the electrophoretic patterns of tumor and control DNA (Supplementary Table S1 is available at Carcinogenesis Online) using seven microsatellite markers (D4Mit98, D4Mit105, D4Mit4345, D4Mit97, D4Mit193 and D4Mit94) as described in the...
Mouse Genome Database. Polymerase chain reaction (PCR) products were separated on 3% agarose gels.

**Deletion analysis**

Deletions involving *Epha7* alleles were studied by real-time quantitative PCR (qPCR) following the indications from D’haene et al. (20). PCRs were carried out with genomic DNA using the LightCycler FastStart DNA MasterPLUS SYBR Green I kit (Roche, Mannheim, Germany). Empirical validation of primers for *Epha7* was performed using dilution series for qPCR assays. Tubulin, beta 5 class I (*Tubb5*) and β2-microglobulin (*B2M*) genes, which are known not to be affected in these tumors, were used as references to accurately measure and correct for variations in the total amount of input DNA in mouse and human, respectively. Designed primers for mouse and human genes are included in Supplementary Table SII, available at Carcinogenesis online. The normalization for DNA deletion was made by comparison with samples exhibiting a known copy number (control thymuses with two *Epha7* alleles and tumor samples exhibiting heterozygous deletion of one allele of *Epha7* gene).

qPCR products were analyzed with SYBR Green using the LightCycler.
technology (Roche) and a later analysis by 2^ΔΔCT method (21). Since it is known that tumor samples could have a minority of contaminating stroma cells, we have considered that values significantly <1 (the value corresponding to a control sample with two Epha7 copies) must be indicative of Epha7 deletion.

Analysis of CpG methylation status
DNA methylation patterns in the CpG islands (CGIs) were determined by bisulfite-mediated conversion of unmethylated cytosines to uracil. Bisulfite genomic sequencing of multiple clones was performed following the detailed protocol described at http://www.epigenome-noe.net/WWW/researchtools/pdfs/p34.pdf. In brief, modification of DNA with sodium bisulfite was carried out as described (22). Then, Methyl Primer Express software (Applied Biosystems, Foster City, CA) was used to design bisulfite-modified DNA-specific primers spanning all the transcription start sites of the genes of interest. Next, DNA amplification was carried out using methylation-specific PCR (22). We also performed a second analysis of the promoter regions under standard PCR conditions, cloning the PCR products into pGEM-TEasy Vector (Promega, Madison, WI), randomly selected 8–10 colonies per sample and performed sequencing using an ABI Prism 3130XL Applied Biosystems DNA sequencer. Finally, we analyzed the methylation of every CpG using Sequencing Analysis 5.2 Software from Applied Biosystems. DNA methylation at each CpG site was determined in at least eight clones by bisulfite sequencing of the forward or reverse strands. A complete table with all the primers used is shown in Supplementary Table SII, available at Carcinogenesis online.

Semi-quantitative reverse transcription–PCR expression analysis
Reverse transcriptions was performed with total RNA treated with DNase I (Ambion, Austin, TX) using oligo(dT) primer and SuperScript II reverse transcriptase (Life Technologies, Carlsbad, CA). Variable number of cycles (20–35) was assayed to determine the most appropriate conditions for obtaining semi-quantitative differences in their expression levels. Reverse transcription–PCR (RT–PCR) primers were designed between different exons to avoid genomic DNA contamination (Supplementary Table SII is available at Carcinogenesis online). PCRs were performed simultaneously with two sets of primers, using glyceraldehyde 3-phosphate dehydrogenase as an internal control to ensure complementary DNA quality and loading accuracy. RT–PCR products were visualized by direct ethidium bromide staining in 1.5% agarose gels.

Gene expression by quantitative RT–PCR
Transcriptional levels of thymus expressing genes were quantified by real-time quantitative RT–PCR using LightCycler (Roche). RT–PCRs were carried out with total RNA using the one-step LightCycler SYBR Green I kit (Roche). The gene-specific primer sequences were designed between different exons to avoid genomic DNA contamination (Supplementary Table SII is available at Carcinogenesis online). Relative expression values were calculated as the messenger RNA amount of each gene relative to that of glyceraldehyde 3-phosphate dehydrogenase (used as reference) and normalized to the relative expression of a non-treated thymus sample, using the LightCycler Relative Quantification software (Roche).
Statistical analyses
The Shapiro–Wilk test was used to check expression data sets for normality, and the Levene test was used for homogeneity of variances. Student’s t-test was used to compare control and experimental groups with Bonferroni correction for multiple comparisons. All statistical tests were carried out using R software (version 2.11.0).

Results
Mouse T-LBLs induced by γ-irradiation exhibit frequent LOH at a region of chromosome 4 orthologous to human 6q15–6q16.3
Previous studies in our laboratory reported frequent LOH in the proximal region of mouse chromosome 4 in T-cell lymphomas induced by γ-irradiation (TLSR5), which includes the syntenic counterpart of human 6q (19). In this work, a detailed map of this region has been assembled in mouse lymphomas to define a region of minimal LOH.

DNAs from 20 T-cell lymphomas induced by γ-rays in (BALB/cJ × C57BL/6J) F1 (BLB) were screened with microsatellite markers covering the entire orthologous region. We found that 20% (4/20) lymphomas induced in BLB hybrid mice exhibited LOH involving seven microsatellites, suggesting the existence of a common LOH region of 9.63 Mb in mouse chromosome 4 (between D4Mit105 and D4Mit94 markers), which is orthologous to human 6q15–16.1 (Figure 1; Supplementary Table SIII is available at Carcinogenesis online).

Gene content of minimal region of deletion and selection of candidate genes
Gene scanning of the mouse LOH region, using the ENSEMBL (www.ensembl.org) and MGI (www.informatics.jax.org) mouse genome browsers, revealed the presence of 13 coding genes as potential targets of this deletion (Figure 1). Although all these genes are expressed in the thymus, only five of them (Manea, Pnrc1, Casp8ap2, Map3k7 and Epha7) showed significant downregulation in mouse T-LBLs induced by γ-irradiation, Epha7 being the one exhibiting the lowest expression levels (Supplementary Figure S1 is available at Carcinogenesis online). Given that Epha7 might be expressed as alternative transcripts with different functional properties (23), we conduct a more detailed analysis of the gene expression patterns using different primers that specifically amplify the main alternative isoforms. We show here that control thymuses are able to express two different transcripts corresponding to the full length and the shorter soluble variant. In contrast, all T-cell lymphomas (31/31), including tumors without LOH, exhibited reduced expression of both isoforms (Figure 2; Supplementary Table SIII is available at Carcinogenesis online).

Mechanisms of gene downregulation
Since LOH may be explained either by uniparental disomy or by allele deletion, we assessed the occurrence of deletions in an extended collection of mouse lymphomas by real-time qPCR using specific primers for Epha7 gene. Intriguingly, only 2 of 31 mouse lymphomas (6.45%) showed Epha7 allele deletions (Supplementary Table SIII is available at Carcinogenesis online).

Although all analyzed tumors showed significant downregulation, only a part of them showed the loss of one Epha7 allele. Thus, we considered the possibility of other inactivating mechanisms. Interestingly, tumor suppressor genes inactivated in T-LBLs induced by γ-irradiation rarely undergo point mutations but are usually inactivated by a combination of deletion and epigenetic mechanisms (24–27). Our results indicated that all candidate genes at TLSR5 harbor CGIs in the proximity of their promoters, but only CGI of Epha7 gene was significantly hypermethylated in a subset of mouse lymphomas (Supplementary Figure S2 and Supplementary Table SIII are available at Carcinogenesis online). To further validate the methylation status of Epha7, we investigated the methylation profile and the level of CGI methylation of this gene. By using a methylation density cutoff of 15%, methylation prevalence of Epha7 was 64.51% (20/31) and...
cinogenesis (20/21; 95.23%), but only a part of them exhibited allele deletion (3/18; 16.66%). In almost all the analyzed primary T-LBLs, (a) Methylation-specific PCR for the EPHA7 gene in a representative sample human T-LBL. The presence of a PCR band under lanes M or U indicates methylated or unmethylated genes, respectively. In vitro methylated DNA (IVD) was used as a positive control for methylated DNA; Normal Lymphocytes (NL) was used as a positive control for unmethylated DNA. (b) Schematic depiction of the EPHA7 CGI around the transcription start site (long black arrow). Short vertical lines represent CpG dinucleotides. The presence of methylated (black square) or unmethylated cytosines (white square) is indicated. Methylation density is indicated as the percentage of methylated CpG sites. From these observations, Epha7 would be downregulated by either LOH (6/31; 19.35%) or promoter hypermethylation (16/31; 51.61%) or a combination of both mechanisms (4/31; 12.90%) and emerges as the best candidate gene for TLSR5 deletion (Supplementary Table SIII is available at Carcinogenesis online).

Studies in human samples

Similar to our results in murine lymphomas, we have found significant downregulation of EPHA7 in almost all the analyzed primary T-LBLs (20/21; 95.23%), but only a part of them exhibited allele deletion (3/18; 16.66%) (Figure 4; Supplementary Table SIV is available at Carcinogenesis online). As occurred in mouse, EPHA7 exhibits a CGI region that was significantly hypermethylated in a subset of human T-LBLs (Figure 5a and b; Supplementary Table SIV is available at Carcinogenesis online). The methylation prevalence was 44.44% (8/18), and the methylation density ranged from 18.30 to 86%. Thus, we conclude that inactivation of EPHA7 in human T-LBLs may occur exclusively by promoter hypermethylation (7/16; 43.75%), although in some tumors, it seems to occur by allele deletion (2/16; 12.5%) or by a combination of deletion and epigenetic events (1/16; 6.25%) (Supplementary Table SIV is available at Carcinogenesis online).

In addition to primary tumors, Jurkat cell line derived from T-ALL patients also exhibited promoter hypermethylation despite not having 6q deletions (Figure 6a and b). To confirm whether promoter DNA hypermethylation is involved in deregulating the expression of EPHA7, Jurkat cells were treated with 5-aza-2′-deoxycytidine (a specific inhibitor of DNA methylation). Significant demethylation was observed after treatment with 5-aza-2′-deoxycytidine (Figure 6a), which brings about EPHA7 upregulation (Figure 6b). The methylation profile and the level of CGI methylation of EPHA7 in Jurkat cells are indicated in Figure 6c.

Discussion

Human 6q deletion is a recurrent cytogenetic abnormality in T-ALL (1,2). Cytogenetic and molecular analyses of 6q in hematological malignancies revealed eight regions of minimal deletion (3,28–34) that have proven to be of great value for defining distinct clinical–pathological subsets of non-Hodgkin’s lymphoma (32). Differences in the common regions with LOH at chromosome 6q have also been used to differentiate pediatric precursor T-ALL and T-LBLs (35).

Concerning candidate genes, a fluorescence in situ hybridization analysis of bone marrow samples from patients with acute lymphocytic leukemia, as well as three cell lines derived from patients with T-ALL, led to the definition of a minimal deletion region between markers D6S1510 and D6S1692 (6q16 band) and the proposal of GRIK2 as the gene most frequently affected in this deletion. In fact, some T-ALL patients with 6q deletion exhibit significant reduction in GRIK2 (3). In contrast with these observations, elevated levels of expression have been found in one acute lymphocytic leukemia patient and in other types of tumors (prostate, kidney, trachea and lung), suggesting that this gene may have a protective effect against tumor development (3). This might be the case with mouse γ-irradiation-induced T-LBLs since the orthologous mouse Grik2 gene maps at chromosome 10 in a region frequently affected by major chromosomal gains (36). Using high-resolution genomic profiling of childhood T-ALL, Remke et al. (4) also proposed CASP9 as another target gene for 6q15–16.1 deletion. In view of the enormous size and complexity of 6q deletion, other candidate genes yet to be studied are quite likely to have a similar effect on T-cell lymphoma development.

Based on the analysis of an animal model and the study of a collection of human T-LBL samples, here, we present evidence for the first time that EPHA7 might be a major factor for genetic susceptibility to T-LBL. The fact that this gene is inactivated preferentially by promoter hypermethylation is in agreement with several reports emphasizing the importance of epigenetic gene inactivation in hematological malignancies (37,38).

Previous studies indicated that the role of this gene in cancer development may be controversial. For example, ALL1 fusion proteins are able to induce upregulation of EPHA7 in human acute leukemias (39), and this gene is usually upregulated in a variety of solid tumors (40–42). In contrast, colon carcinomas showed reduced EPHA7 expression (40,43) and bone metastasis from prostate cancer showed complete absence of EPHA7 expression (44). Furthermore, differential expression ranging from overexpression to downregulation has been found in gastric carcinoma (45).

Evidence of hypermethylation at its promoter region has been reported in mouse and human B-cell lymphomas (23) and in several solid tumors, such as colorectal cancer (43) and prostate cancer (46). Interestingly, the pattern of DNA hypermethylation we have observed in T-cell lymphomas is very similar to those reported for B-cell lymphomas and colorectal cancer, suggesting that a common methylation profile is shared by different type of tumors.
The effect of EPH receptor A7 gene on tumorigenesis has been described in detail and is not further analyzed here. EPHA receptors have been shown to regulate cell survival, migration and cell–cell and cell–matrix interactions (47). In thymocytes, EPHA receptors have been proposed to inhibit anti-CD3-induced apoptosis of CD4+CD8+ cells. Furthermore, they also inhibit T cell receptor activation of the Ras-mitogen-activated protein kinase pathway (48,49). Since pioneer molecular studies on γ-radiation-induced T-cell lymphomas led to the identification of N-ras and K-ras as critical oncogenes in these tumors (17,18,50), it is reasonable to think that activating Ras mutations are probably a way whereby tumor cells avoid inhibition by EPHA7.

Taken together, all these observations lead us to propose that EPHA7 may be considered as a new tumor suppressor gene for this region. Since other genes, as GRIK2 (6q16.3) and CASP8AP2 (6q15–16.1), have also been identified in acute lymphocytic leukemia, del6q seems to be a complex region where inactivation of multiple genes may cooperatively contribute to the onset of T-cell lymphomas.

Supplementary material

Supplementary Tables SI–SIV and Figures S1–S2 can be found at http://carcin.oxfordjournals.org/.

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