Epigenetic silencing of E- and N-cadherins in the stroma of mouse thymic lymphomas


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A number of papers have shown that alterations of several oncogenes and tumour suppressor genes expressed by thymocytes help explain the development of this kind of lymphomas (1–12). Still the thymus is a heterogeneous lobed organ having also stromal cells that provide a variety of microenvironments where thymocytes proliferate and mature (13). As of this writing there are no reports recording genetic alterations of critical genes expressed by stromal cells during the origin and/or progression of thymic lymphomas.

In order to characterize the pattern of expression of some of these genes, our research focused on E- and N-cadherin that are expressed by stromal epithelial and mesenchymal cells, respectively. Classical cadherins encode for a group of membrane receptors that mediate calcium-dependent homotypic (and sometimes heterotypic) cell-to-cell adhesion. These proteins might be involved in the maintenance of haematopoietic stem cells, acting as a negative regulator of WNT-pathway signalling by binding thereby and sequestering β-catenin from the nucleus. Alternatively, the accumulation of β-catenin in the cytoplasm may facilitate its displacement to the nucleus, where it would bind to the transcriptional factor Tcf thereby stimulating transcription of target genes involved in cell proliferation and differentiation (13–15).

In mice, both stromal epithelial cells as well as fetal thymocytes and a fraction of the neonatal and adult T-cells clearly express E-cadherin (16, 17). Interestingly, the progression of double-negative (CD4−CD8−) to double-positive (CD4+CD8+) thymocytes may be disturbed after inhibition of the homotypic E-cadherin interactions (18). Furthermore, interactions of thymic epithelial cells (expressing E-cadherin) with CD103+ thymocytes [expressing α6(1CD103)β7 integrin] can lead to enhanced thymocyte proliferation in human (19). Several other classical cadherins (Cadherin-6, 8 and 11) have been found by RT–PCR on murine thymocytes, though these studies have not been confirmed on immunological ground (17). In human bone marrow N-cadherin is expressed by early haematopoietic progenitor cells (CD34+CD19+) but is down-regulated by more mature progenitor cells (20).

In oncogenesis, both a loss of the epithelial E-cadherin (Cdh1) and a gain of the mesenchymal cadherins such as N-cadherin (Cdh2) have been reported during the progression of many solid tumours (14). Still, the role of cadherins in primary haematopoietic malignancies is only starting to be recognized (21–24). For this reason, it is reasonable to think that the study of these members of the cadherin family in the thymus may help us reach a more precise knowledge of the molecular alterations underlying the progression of thymic lymphomas.

In this study, we report that in the thymus of mice E-cadherin is essentially expressed by stromal cells and to a lesser extent by thymocytes, whereas N-cadherin is only expressed by stromal cells. In addition, the analysis of a sample series of γ-radiation-induced murine-thymic-lymphomas revealed significant reductions of both E- and N-cadherin in all tumours examined suggesting the existence of epigenetic

Introduction

Gamma-radiation-induced thymic lymphomas consist of a heterogeneous group of T-cell lymphoblastic lymphomas characterized by an uncontrolled expansion of immature T-cell precursors that fail to complete differentiation.
control events. For such reasons, we propose that the down-regulation of stromal E- and N-cadherin in these cells may be contributing to the development of primary thymic lymphomas.

Materials and methods

Mice and tumour induction

Reciprocal inter-strain crosses, involving males and females from the BALB/c and C57BL/6 strains, were carried out in order to generate F1 hybrid mice. Five adult mice were treated with a single high dose of γ-radiation (10 Gy) and killed 24 h later. A series of 14 thymic lymphomas were obtained by whole-body γ-radiation of 17 adult mice split into four weekly doses of 1.75 Gy as described in a previous work (25). Five additional thymic lymphomas were induced in C57BL/6 mice. All of the tumour samples were frank T-cell lymphomas in their most advanced stage of development.

Sample cell fractionation

Samples from three thymic lymphomas, two thymuses of mice treated with a single high dose and three control-healthy thymuses from C57BL/6 mice were washed and strained through a nylon mesh (BD Falcon Cell Strainer, BD Biosciences, Belgium). Stromal-enriched cell fractions were obtained by collagenase digestion according to a previously described method (26). This procedure allowed for the purification of a significant amount of stromal cells (including epithelium, endothelium, reticular fibroblasts, macrophages, dendritic cells and neuroendocrine cells) from other cells (CD45+) derived from the haematopoietic stem cells. Thymocyte isolation was carried out by centrifugation on Ficoll-Hypaque (Pharmacia Biotech, Upsala, Sweden) as described previously (27). Thymocytes were afterwards positively selected by magnetic sorting using the Pan T-cell Isolation kit (Miltenyi Biotec GmbH, BG, Germany). The purity of the isolated thymocytes was confirmed through flow cytometry (CytoFACS 500 Series Flow Cytometry Systems, Beckman Coulter, Fullerton, CA) using fluorescein isothiocyanate (FITC)-conjugated anti-CD8 (Becton Dickinson, Pharmingen), phycoerythrin (PE)-conjugated anti-CD4 (Becton Dickinson, Pharmingen) and FITC-conjugated anti-CD45 (Becton Dickinson, Pharmingen).

Histopathology and immunofluorescence staining

For histopathology two control thymuses from C57BL/6 and two radiation-induced thymic lymphomas were fixed with 4% formaldehyde in phosphate-buffered saline (PBS) overnight at 4°C, and embedded in paraffin. Tissue sections were prepared, stained with haematoxylin and cosin and examined under the microscope. Immunofluorescence staining was also performed on fine tissue sections (2 μm) of control thymuses and thymic lymphomas using mAb to E-cadherin (1:50, Becton Dickinson, Pharmingen). The secondary antibody used was Alexa 488 (Molecular probes, Eugene, OR). The sections were mounted on Vectashield (Vector Laboratories Inc, Burlingame, CA) and the preparations were visualized using a Olympus photomicroscope IMT-2, equipped with a HBO 100 W mercury lamp. The corresponding filter sets for fluorescein microscopy use were ultraviolet (UV, 365 nm, exciting filter UG-1) for 4′,6-Diamidino-2-phenylindole (DAPI) (Roche) and blue (450–490 nm. Exciting filter BP 490) for Alexa.

Quantitative real-time RT–PCR

For the determination of gene expression levels of N- and E-cadherin, as well as those of Snail1, a quantification assay on the basis of real-time reverse transcription PCR with a LightCycler instrument (Roche Diagnostics, Penzberg, Germany) was established, by means of a fluorescence-resonance energy transfer (FRET) technique. This method involves two oligonucleotide probes that bind to the target DNA. In our case the 3′ probe had a donor fluorophore (fluorescein) at the 5′ end whereas the 5′ probe had an acceptor fluorophore (LightCycler-Red 640 or LightCycler-Red 705) at the 3′ end. RT–PCR of the gene encoding the tubulin beta 5 chain (Tubb5) (http://www.informatics.jax.org) was used as an internal control of the RNA quality and its amplification. Primers for mRNA amplifications and for the FRET probes were as follows: for Tubb5, 5′-AAAGCTTCTGAGGCAAATC-3′ (forward), 5′-GGCTTTTGGCCGCACTGCTTGT-3′ (fluorescein) and 5′-GCCGACGACGTACGTTAATC-3′ (reverse); for Snail1, 5′-CCTCATAATGCTTTTCCAGG-3′ (forward), 5′-CTCTGAACTTCCTTCCTGAAATC-3′ (reverse) and 5′-ACTGTATATCTCCTACATCCAGT-3′ (fluorescein) and 5′-GAGAAGACCATCTTGTTCCTCCAC-3′ (LCRed640); and for Tubb5, 5′-GGTCAC-
Results

Histopathological analysis
As reported by other studies, control thymuses from C57BL/6J mice exhibit a clear distinction between the cortex and the medulla (Figure 1A–C). In thymus lymphoma the gland is considerably larger and the histopathological sections show a profound disorganization with a poor demarcation of the cortical and medullar areas featuring large lymphoblastic T-cells, frequent mitoses and pycnotic cells (Figure 1D and E). Finally, stromal cells that are visible in the medulla of control thymuses (Figure 1C) appear randomly distributed in thymic lymphomas (Figure 1E).

Expression analyses of E-cadherin and N-cadherin
The expression of E-cadherin in the thymus was initially determined by immunofluorescence staining (Figure 2). In control thymuses (Figure 2A and E) strong surface labelling was found essentially in the medullar-stromal cells and to a lesser extent in some of the cortical epithelial cells (Figure 2E). In thymic lymphoma immunoreactivity appears to be restricted to aggregations of stroma-tumoral cells randomly dispersed (Figure 2B and F).

Since immunofluorescence staining is not sensitive enough to exclude the possibility that thymocytes could also express E-cadherin, we determined which cell types really expressed this gene through quantitative fluorescent-real-time RT–PCR, using total RNA from whole-control thymuses and separated cell fractions. Taking the level of expression of E-cadherin in control thymuses as a reference unit, similar amounts (200 ng) of total RNA from the stromal cells exhibited the highest levels of mRNA expression (mean normalized value 4.46 and SD 0.12), whereas purified thymocytes evidenced significant but substantially lower levels of mRNA from this gene (mean normalized value 0.19 and SD 0.022). Using a similar approach, we demonstrated that N-cadherin is also expressed by the stromal cells of control thymuses (mean normalized value 3.88 and SD 0.56). In isolated thymocytes mRNA of N-cadherin was practically undetectable (mean normalized value 0.03 and SD 0.03).

Since loss of epithelial E-cadherin expression and gain of mesenchymal N-cadherin have been observed during the development of many solid cancers, we further investigated whether such changes could also be operating in primary thymic lymphomas induced with γ-irradiation. To this end, we performed quantitative fluorescent-real-time RT–PCR experiments using total RNA extracted from the thymuses of five mice 24 h after treatment with a single high dose of radiation, as well as from 14 thymic-lymphoma-bearing mice treated weekly with four low doses of radiation, and thymuses of three treated mice that did not develop thymic lymphoma. A significant increase in the expression of both E- and N-cadherin for thymuses of mice treated with single high doses when compared with control thymuses was found (E-cadherin: mean normalized value 35.17; SD 0.216; N-cadherin: mean normalized value 32.47 and SD 0.38). In the case of E-cadherin this increase can be attributed to both stromal cells and thymocytes (Stroma: mean normalized value 64.56, SD 0.14; Thymocytes mean normalized value 6.51, SD 0.47). However, the expression of these genes remained unaltered in tumour-free mice (E-cadherin mean normalized value 0.96, SD 0.09; N-cadherin mean normalized value 0.95,

Epigenetic down-regulation of cadherins in thymic lymphomas

Fig. 1. Tissue sections from a control thymus (A–C) and a thymic lymphoma (D–E) stained with hematoxylin and eosin. Sections of control thymus show clearly defined cortical (C) and medullar (M) regions, with the thymocytes mainly located within the cortex in the spaces created by the epithelial cells. In thymic lymphoma no apparent differences can be detected between the cortex and the medulla, and thymocytes are randomly distributed. Scale bar: 200 μm (A and D) and 10 μm (B, C and E).
In relation to thymic lymphomas all these tumours exhibited a considerable reduction in the levels of E-cadherin expression in comparison with control thymuses (mean normalized value 0.16 and SD 0.20), this reduction being produced by down-regulation of this gene in the stromal portion of lymphomas (Stroma: mean normalized value 1.62, SD 0.18). On the other hand, the levels of expression of N-cadherin (Cdh2) were likewise considerably reduced in 13 of the 14 tumours (i.e. 92.85%) (mean normalized value 0.47; SD 0.32). Table I shows specific data about mRNA expression of E- and N-cadherin in each tumour. E-cadherin values varied between 0.025 and 0.695 (SD from 0.007 to 0.148) in relation to control thymuses. The levels of N-cadherin expression ranged from 0.145 to 0.73 (SD from 0.007 to 0.078) in relation to the mean value from control thymuses. All RT–PCR data represent an average from two independent amplifications for each sample. A one-way ANOVA and a Tukey comparison post-test evidence that differences between control thymuses and thymic lymphomas were clearly significant ($P < 0.001$).

In order to determine whether the mRNA profiles of thymic lymphomas correlated well with protein expression western blot analyses on the same panel of tumours were performed. Using the expected molecular weights as well as the positive controls from HaCaT cells and brain samples as a reference, we deduced that all of the tumours have failed to express significant amounts of proteins from both of these genes. Whereas 2 out 14 tumours (14.28%) exhibited traces of E-cadherin, this protein was completely absent in 12 out of the 14 tumours (85.72%) (Table I and Figure 3A). Similar results were obtained for N-cadherin protein expression (Table I and Figure 3B). The expression of E-cadherin was also studied using protein samples from stromal cells and isolated thymocytes. Consistent with the transcriptional data, the amount of protein detected in stromal cells of three of the tumours was found to lie between those observed in whole control thymuses and those of thymic lymphomas (data not shown).

**Hypermethylation in tumour samples**

Previous works have demonstrated that E-cadherin is frequently silenced by promoter hypermethylation in many other cancer types (29). For such reasons we were interested...
Epigenetic down-regulation of cadherins in thymic lymphomas

Table I. Gene expression and methylation data of thymic lymphomas

<table>
<thead>
<tr>
<th>Sample</th>
<th>RT–PCR (LightCycler)</th>
<th>WB</th>
<th>MSP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cdh1 Normalized mean ratio ± SD</td>
<td>Cdh2 Normalized mean ratio ± SD</td>
<td>Snai1 Normalized mean ratio ± SD</td>
</tr>
<tr>
<td>(C57BL/6Jx BALB/cJ) F1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>BLB 4</td>
<td>0.06 ± 0.0141</td>
<td>0.145 ± 0.0071</td>
<td>0.525 ± 0.0212</td>
</tr>
<tr>
<td>BLB 6</td>
<td>0.045 ± 0.0071</td>
<td>0.705 ± 0.0354</td>
<td>0.48 ± 0.0141</td>
</tr>
<tr>
<td>BLB 18</td>
<td>0.095 ± 0.0212</td>
<td>0.475 ± 0.0212</td>
<td>0.68 ± 0.0283</td>
</tr>
<tr>
<td>BLB 31</td>
<td>0.695 ± 0.1485</td>
<td>0.73 ± 0.0424</td>
<td>1.875 ± 0.0778</td>
</tr>
<tr>
<td>BLB 36</td>
<td>0.045 ± 0.0071</td>
<td>0.29 ± 0.0141</td>
<td>0.54 ± 0.0283</td>
</tr>
<tr>
<td>BLB 44</td>
<td>0.05 ± 0.0141</td>
<td>0.3 ± 0.0141</td>
<td>0.78 ± 0.0283</td>
</tr>
<tr>
<td>BLB 50</td>
<td>0.55 ± 0.1131</td>
<td>0.72 ± 0.0424</td>
<td>1.01 ± 0.0424</td>
</tr>
<tr>
<td>BLB 60</td>
<td>0.025 ± 0.0071</td>
<td>0.22 ± 0.0141</td>
<td>0.61 ± 0.0283</td>
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<tr>
<td>BLB 66</td>
<td>0.14 ± 0.0283</td>
<td>0.385 ± 0.0212</td>
<td>0.565 ± 0.0212</td>
</tr>
<tr>
<td>BLB 67</td>
<td>0.165 ± 0.0354</td>
<td>0.475 ± 0.0212</td>
<td>0.545 ± 0.0212</td>
</tr>
<tr>
<td>BLB 74</td>
<td>0.21 ± 0.0424</td>
<td>0.26 ± 0.0141</td>
<td>1.76 ± 0.0707</td>
</tr>
<tr>
<td>BLB 85</td>
<td>0.07 ± 0.0141</td>
<td>1.385 ± 0.0778</td>
<td>1.76 ± 0.0707</td>
</tr>
<tr>
<td>BLB 88</td>
<td>0.095 ± 0.0212</td>
<td>0.395 ± 0.0212</td>
<td>1.175 ± 0.0495</td>
</tr>
<tr>
<td>BLB 95</td>
<td>0.06 ± 0.0141</td>
<td>0.19 ± 0.0141</td>
<td>0.975 ± 0.0354</td>
</tr>
</tbody>
</table>

A summary of the results derived from transcriptional expression (RT–PCR), translational expression (WB) and the methylation status of promoter regions (MSP) of cadherin genes, as well as RT–PCR analysis of Snai1. The WB values for both genes were designed as + (similar level of expression than in the controls), (–) (reduced expression). An asterisk over a (–) sign denotes traces of expression. M, methylated; U, unmethylated.

N-cadherin promoter hypermethylation was detected in 12 out of 14 (i.e. 85.71%) of the analyzed tumours (Figure 5A and Table I). As in the previous case, a sequencing analysis using external primers corroborated these results, with 19 methylated CpG dinucleotides around the transcription start site (Figure 5B). All of the 12 methylated tumours exhibited reduced levels of mRNA transcription while one of the two un-methylated tumours expressed detectable levels of expression (in fact, similar to or even higher than those of the control samples) (Table I). Interestingly, we found a significant decrease in the level of expression of this gene between methylated and unmethylated tumours (Student’s t-test, P < 0.05).

Fig. 3. Western blot analysis of E-cadherin (A) and N-cadherin (B). B6 and Bal identified the C57BL/6J and BALB/cJ strains, respectively. F1, control-hybrid mice. BLB4-95, thymic lymphomas induced in F1 hybrids. HaCaT and Brain, positive-control samples. Tumours BLB18 and 66 exhibit traces of E-cadherin protein and tumours BLB66 and 74 exhibit traces of N-cadherin protein.
have not detected significant changes in the status of promoter methylation in any case with respect to the control samples (data not shown).

Transcriptional expression of Snai1
As 10 out of the 14 thymic lymphomas exhibited reduced mRNA E-cadherin expression but did not display promoter hypermethylation, we finally tested whether these tumours exhibited an increased expression of Snai1, a gene capable of inhibiting E-cadherin (39). Interestingly, three of these tumours showed an over-expression of Snai1 (Table I), suggesting that this gene could be responsible (at least in part) for the down-regulation of E-cadherin in some of the thymic lymphomas.

Discussion
Classical cadherins have been involved in the maintenance of haematopoietic stem cells, as well as in thymocyte differentiation (18) and proliferation (19). In regard to oncogenesis, the majority of the studies have focussed on the diminished expression of E-cadherin from different carcinomas (29).

With the exception of pancreatic-ductal-epithelial cell lines (30), the reduced level of E-cadherin mRNA observed in several human cancers (including melanoma, prostate and breast cancers) is often accompanied by the de novo expression of N-cadherin (31,32). In fact, N-cadherin is up-regulated in the more invasive and less differentiated of the breast cancer cell lines that lack E-cadherin expression (33,34). These observations led to postulate for a ‘cadherin switch’ between E- and N-cadherin during the progression of cancer, suggesting that the loss of epithelial E-cadherin is followed by an up-regulation of the mesenchymal N-cadherin that enhances the motility and invasive capacities of the tumorous cells. Recent insights into haematological malignancies have revealed a significant reduction or an absence of E-cadherin expression in human acute myelogenous leukaemia and in chronic lymphocytic leukaemia (21) as well as in Hodgkin and Red-Stenberg cells (22). In humans, N-cadherin has also been detected in T-cell leukaemia and lymphoma cells but not on normal leukocytes (23,24). To this day, however, no one has reported anything on the role played by these cadherins in primary thymic lymphomas.
Using immunofluorescence and quantitative fluorescence-real-time RT–PCR experimental approaches, we confirmed that stromal cells of control thymuses express significant amounts of E-cadherin mRNA and demonstrated for the first time that the stromal cells likewise express N-cadherin mRNA even though at more reduced levels. Isolated thymocytes also expressed small amounts of E-cadherin mRNA yet failing to produce detectable levels of mRNA from the N-cadherin gene.

As expected, the analysis on a sample series of advanced murine thymic lymphomas induced by γ-rays evidenced a significant reduction of both the E- and N-cadherin expression in almost all of the tumours, suggesting that inhibition of both genes may be critical in the progression towards the most advanced stages of lymphomagenesis. These hypotheses might be supported by the fact that in thymuses from treated-lymphoma-free mice the levels of E-cadherin did not differ significantly when compared with those detected in control non-treated thymuses. However, it should be stressed that thymuses from mice exposed to single high doses of radiation and killed 24 h later experienced a considerable over-expression of both genes. This early response to radiation could be indicating that, contrary to the situation recorded in the most advances stages, high levels of E- and N-cadherin may be favouring the initial stages of tumorigenesis. This is probably accomplished by enhancing thymocyte cell proliferation through adhesive interactions between thymic epithelial cells and thymocytes mediated by the E-cadherin-CD103 integrin-ligand-pair (19).

With regard to thymic lymphomas down-regulation of both genes appears to occur concomitantly with the progression towards the most advances stages of lymphomagenesis.

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**Fig. 5.** Methylation specific analysis at the promoter region of the N-cadherin gene. B6 and Bal identify the C57BL/6J and BALB/cJ strains, respectively. F1, control-hybrid mice. BLB4-95, thymic lymphomas induced in F1 hybrids. (A) MSP of a representative CpG island at the promoter region. U, unmethylated; M, methylated. (B) Determination of cytosine methylation at specific CpG sites by DNA sequencing of bisulfite-treated genomic DNA from control and tumours. Open and filled circles denote non-methylated and methylated CpG sites, respectively.
In contrast to peripheral lymphomas, these results suggest that in primary thymic lymphomas N-cadherin is not countering the effects produced by E-cadherin. Whereas the up-regulation of N-cadherin might prove useful to promote invasivity and metastasis in peripheral lymphomas, a completely different scenario seems to be operative in thymic lymphomas where E- and N-cadherin apparently work in the same direction. Reduced levels of N-cadherin expression are in agreement with the loss of heterozygosity we have already detected in other sample series of this kind of tumours involving the chromosomal region where this gene is located (34). Since both E- and N-cadherin are essentially expressed by stromal cells, the detection of significant differences between their levels of expression in stromal cells of control thymuses versus thymic lymphomas lead us to postulate that the decline in the levels of expression of both genes in whole lymphomas could be tentatively attributed to their down-regulation in stromal cells.

To gain further understanding of the mechanisms involved in the down-regulation of cadherins, the levels of methylation of their promoter CpG islands by means of a MSP/sequencing analysis were studied. The importance of gene silencing in γ-radiation-induced thymic-lymphomas by promoter hypermethylation is highlighted in previous reports that call upon epigenetic events in the inactivation of other genes (p15/Ink4b and p16/Ink4a) (2,3,5). Hypermethylation of the E-cadherin promoter region has been reported in several kinds of human tumours including breast, gastric, and thyroid carcinomas, oral squamous cell carcinoma, hepatocellular, prostate and nasopharyngeal carcinomas, as well as in cervical cancer cell lines and primary cervical cancers, suggesting that promoter hypermethylation is one of the main mechanisms involved in E-cadherin silencing during tumour progression (29,36). However, inactivation of N-cadherin through epigenetic mechanisms had been only described in human pancreatic cells (30).

Our results link the N-cadherin gene as a common target for transcriptional inactivation by promoter hypermethylation, whereas only a minor fraction of these tumours (4/14) seems to inactivate E-cadherin by this same procedure. For this reason, alternative mechanisms such as mutations or epigenetic alterations favoured by the over-expression of specific transcriptional repressors or loss of trans-activating proteins without apparently increasing CpG promoter methylation should also be involved in E-cadherin inactivation (14,37–39). For this reason, we have also studied the pattern of transcriptional expression of Snail, one of the transcriptional repressors that may inactivate E-cadherin through the recruitment of a histone–deacetylase complex to the unmethylated Cdh1 promoter (40). Though some apparently contradictory results have been described, Snail appears to be a candidate repressor of E-cadherin in several solid tumours such as breast and gastric carcinomas as well as in hepatocarcinomas (41). Interestingly, it was found that a fraction of the thymic lymphomas exhibiting a reduction in the levels of E-cadherin mRNA not accompanied by promoter hyper-methylation did show an over-expression of Snail. Such over-expression of Snail in these tumours might therefore be involved in the inactivation of E-cadherin in these tumours through promoter-histone-deacetylitation without promoter DNA-hypermethylation. Interestingly, we have not observed significant changes in the pattern of methylation at the promoters of E- and N-cadherin genes in tumour-free mice after aging or radiation treatments, suggesting that the observed methylation and expression changes detected in tumours appears to be the consequence of tumorigenesis.

These results do not preclude the existence of a possible cooperation of different repressors in E-cadherin regulation. In addition, there remain seven lymphomas for which the inhibition of E-cadherin remains to be explained. Although, we tried to analyze the expression of other transcriptional inhibitors such as Slug (Snai2), E47 and E12 (Tcfε2α) through RT–PCR we have been unable to detect their expression in neither control nor tumorous samples (data not shown). More recently some cis-regulatory elements have been described in the intron 2 of E-cadherin that seems to be essential for gene expression during mouse embryonic development (42).

Taken together these results suggest that γ-radiation-induced thymic lymphomas might result not only from gene alterations occurring in the thymocytes but also from epigenetic alterations involving other genes expressed in stromal cells, thus highlighting the importance of thymus-microenvironment disturbance in the development of these kind of tumours (i.e. the tumour-associated alterations are not in the tumour cells per se, but in the stromal cells). Although there is precedent for this (43), to the best of our knowledge this has been not reported in lymphomas. We propose that an early over-expression of both E- and N-cadherin in response to single high doses of radiation may be favouring the initial stages of limbomagenesis probably by enhancing thymocyte proliferation. However, the histopathological disorganization that occurs in the structure of the frank lymphomas in the most advanced stages of tumorigenesis could, at least in part, be attributed to the epigenetic down-regulation of both E- and N-cadherin. In this regard, we have recently demonstrated that new drugs with DNA demethylating activity, such as zebularine, are able to restore E-cadherin expression and at the same time are effective against the development of the described mouse T-lymphomas (44).

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