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Differential Regulation of Notch Signal Transduction in Leukaemia and Lymphoma Cells in Culture

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Abstract The transduction of Notch signal plays an intricate role in cell differentiation and pathogenesis of haematological malignancies as well as in certain congenital conditions. We found no genomic changes in either gene in 34 leukaemic samples and 25 leukaemia and lymphoma cell lines. The functionality of Notch signalling was tested using *HES1* gene activation. We show that Notch signalling is differentially regulated in T-acute lymphoblastic leukaemia (ALL) and B-lymphoma cells. The Notch pathway is intact in a majority of B-lymphoma cell lines, but EBNA2, which mimics notch function, can occasionally activate the pathway. In contrast, the Notch pathway is constitutively active in T-ALL. This is the first demonstration of a distinction between B-lymphomas and T-cell leukaemias in the functioning of the Notch-signalling pathway. This might be related to their pathogenesis. J. Cell. Biochem. 88: 569–577, 2003.

Key words: leukaemia; lymphoma; notch signalling; HES1; EBNA

The Notch signalling pathway plays a major role in the differentiation of many cell types as well as in the pathogenesis of haematological malignancies such as leukaemias and lymphomas, congenital autosomal dominant condition known as the Alagille syndrome and in cerebral autosomal dominant arteriopathy with subcortical infracts and leukoencephalopathy (CADASIL). Notch1 plays an essential role during embryogenesis [Swiatek et al., 1994], specifically being implicated in myogenesis and neurogenesis [Nye et al., 1994; De la Pompa et al., 1997]. Its role is fundamental to the hematopoietic process, yet it is not fully understood. Notch is expressed in the human bone marrow haemopoietic precursor cells and may be involved in the renewal and differentiation of these cells. It is able to inhibit the differentiation of haemopoietic cells into myeloid lineage [Milner et al., 1996] and is involved in determining lymphopoietic lineage.

Four notch genes viz. *Notch1/TAN-1*, *Notch2*, *Notch3* and *Notch4/int-3* have been identified and these are expressed in unique developmental patterns [Williams and Lardelli, 1995; Lindsell et al., 1996]. The Notch proteins bind their ligands in a non-preferential fashion. The DSL (Delta/Serrate/Lag2) family of genes encode for these ligands, which include the vertebrate homologues of Jagged1 and Jagged2

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[Lindsell et al., 1995; Valsecchi et al., 1997] and Delta1, Delta2, Delta 3 and Dll4 [Joutel and Tournier-Vasserve, 1998; Gray et al., 1999; Shutter et al., 2000].

The expression of Notch and its ligands is upregulated in many human neoplasms. T-cell lymphoblastic leukaemia/lymphoma as well as the Alagille disorder are known to accompany mutations in *Notch1*, *Notch3*, and in *Jagged1* gene that encodes a Notch ligand [Joutel and Tournier-Vasserve, 1998].

In certain T-cell lymphoblastic leukaemias a t(7;9) (q34;q34.3) translocation has been reported. The *Notch* gene was originally identified at this chromosomal translocation breakpoint. Indeed, the breakpoint occurs within 100 bp of an intron in *Notch*, and the translocation seems to lead to the generation of a truncated *Notch* transcript [Ellisen et al., 1991]. This truncated Notch protein appears to be constitutively activated and is capable of transforming cells in vitro [Capobianco et al., 1997].

The Notch family genes encode highly conserved transmembrane proteins, composed of an extracellular ligand binding domain (EC) and a transmembrane signalling domain (IC). Notch signalling involves the proteolytic cleavage and translocation of the intracellular domain into the nucleus. This process has been shown to result in Notch activation [Rand et al., 2000]. This has led to the suggestion that the IC domain is constitutively active and that it is inhibited by interaction with the EC domain. The release of the EC relieves this inhibition and activates Notch signalling. Two mutations of the Notch protein have been described recently, which lead to the formation of structurally distinct Notch extracellular and intracellular domain proteins, and both mutations seem to lead to the shedding of the extracellular domain. These might participate in the development of leukaemia [Hoemann et al., 2000]. All these findings suggest that a deregulation of the Notch signal transduction pathway might lead to neoplastic development.

Notch signalling has other ramifications by being able to interact with other developmentally regulated signal transduction pathways. The Notch signalling pathway interacts with the wnt/β-catenin and Ras pathways of signal transduction, which has been elucidated to some degree [Miller et al., 1999; Fitzgerald et al., 2000].

Recently Cattaneo et al. [2000] have reported a differential expression of *TAN-1*, the human homologue of Notch1 in tumour cells, which suggests that Notch regulation might be an important factor in neoplastic development. We have therefore investigated possible genomic changes associated with *TAN-1* in a number of leukaemia and lymphoma cells, but have found no alterations in its genomic structure.

The HES family of proteins is basic helix-loop-helix transcriptional repressors homologous to the *Drosophila* hairy/enhancer of split proteins. These proteins are the primary down-stream targets of Notch. Notch IC is a potent activator of the promoters of HES family proteins [Beatus et al., 2001; Iso et al., 2001a,b]. HES1 mediates the phenotypic effects by activation of Notch proteins that negatively regulate the differentiation of endocrine cells [Jensen et al., 2000], and inhibit neuronal differentiation [Kageyama and Ohtsuka, 1999]. CSL family proteins are the nuclear mediators of Notch signalling. Mutation of CSL abrogates Notch signalling [Reizis and Leder, 2002]. However, in certain systems CSL has been shown to mediate transcription independently of activation by Notch [Tan and Kadesch, 2001]. Notch signalling is circumvented by the intervention of the Epstein-Barr virus nuclear antigen, EBNA2, which is known to activate HES1. The absence of genomic abnormalities does not guarantee that the signalling pathway was functioning normally. We have therefore also investigated integrity of the Notch signal transduction pathway by examining the expression of *Notch*, *HES1-1* as well as the *EBNA2* status in leukaemic cells in culture with a view to determining the significance of Notch signalling in the development of leukaemias and lymphomas.

MATERIALS AND METHODS

Leukaemic Samples and Cell Lines

The present study examined possible abnormalities in genes associated with the Notch signalling pathway by Southern blotting. We tested 34 DNA samples from leukaemia patients and five cell lines derived from patient material. Of these, 20 were acute myeloid leukaemia primary samples, and 19 T-acute lymphoblastic leukaemia samples (14 primary samples and five lines: FRO*, CEM*, SupT1*, MOLT4*, KE37*).

Lymphoma Cell Lines

We also tested 25 DNA samples obtained from lymphomatous cell lines, as given below:

- Burkitt lymphoma (BL) (four established cell lines: BL135*, RAJI*, EB3, and DAUDI*)
- Sporadic BL (four cell lines: JD38, EW36*, BL41*, and KK124)
- AIDS related BL (three lines: ESIII, AS283A*, and HBL-1)
- Large cell diffuse lymphoma (five cell lines: VAL*, RCK8, BJAB, LY7, and LY8*)
- Hodgkin lymphoma (two lines: KMH2 and HDLM2)
- Large T-cell diffuse lymphoma (two lines: LY12 and LY12.3)
- Primary effusion lymphoma (five lines: BC1, BC2*, BC3, BCP1, and BCBL1).

Southern blot experiments were performed for all the cell lines and cell samples; Northern blot experiments were performed only on cell lines marked with the asterisk. For both Southern blot and Northern blot experiments, we used peripheral blood mononuclear cells (PBMC) from two different healthy donors as controls. The cell lines were cultured in 5% CO_2 atmosphere in complete RPMI supplemented with 10% foetal calf serum. All primary samples were separated on a Ficol gradient.

Molecular Hybridization

For Southern blotting, DNA extraction was performed using standard method [Sambrook et al., 1989]. Fifteen micrograms of DNA from each sample were digested with EcoRI or BamHI. Digested DNA was electrophoresed and blotted on a nylon filter. Hybridization was performed at high stringency using probes for TAN-1.

We employed two different Notch1/TAN-1 probes [described below] for Southern and Northern blot analysis as reported in the section "Retrotranscription and amplification." The human *TAN-1* gene probe employed in Southern blotting was designed to correspond with the gene sequence that overlaps the translocation break point in the extracellular domain of the TAN-1 protein that gives rise to the activated form. Precise information about length and conditions of amplification are given below.

For Northern blotting, total RNA was extracted [Chomczynski and Sacchi, 1987]. Thirty micrograms of DNA from each sample were electrophoresed and blotted on a nylon filter. Hybridisation was performed at high stringency with probes corresponding to the intracellular domain of *TAN-1* to identify possible intracellular activated form, and a part of the coding sequence of *HES-1*. The probes were amplified by reverse transcriptase-polymerase chain reaction (RT-PCR) in the conditions described.

The filters were autoradiographed and hybridisation signals were captured by Kodak DC290 Zoom Digital Camera. The signals were quantified by using Kodak 1D image Analysis Software. The values given in the Table I are ratios of pixels representing the whole area of the target gene signal/18S RNA probe signal. 18S RNA probe was purchased from New England Biolabs.

Retrotranscription and Amplification

PCR was employed to prepare the molecular probes listed below for Northern and Southern blot. PCR was also employed to analyse the status of EBNA2 gene to assess its presence in the cell genome and RT-PCR was used to perform a semi-quantitative analysis of its expression.

Molecular Probes

TAN-1 probe for Southern blot corresponded to 1548 bp fragment spanning exactly from nt 3810 to 5358 of the coding region. The *TAN-1* probe used for Northern blot spanned from nt 6408 to 7202 and *HES1* from nt 922 to 1306 (gene bank NM_005524).

All the DNA fragments used as probes resulted from retrotranscription of 1 μg total RNA extracted from SupT1 cell line and retrotranscribed by M-MLV Reverse Transcriptase (Gibco BRL-Life Technologies) in the conditions suggested. The synthesised cDNAs were diluted 1:500. PCR was performed in a 100 μl reaction mixture containing 10 μl of cDNA or 500 ng of genomic DNA, 10X PCR Buffer II, 12.5 mM $MgCl_2$, 200 μM dNTPs, 1.25 units of AmpliTaq Gold DNA Polymerase (Perkin-Elmer Corporation).

Amplifications were performed, after an initial denaturation at 95°C for 12 min, for the

TABLE I. Expression of Component Genes of the Notch Signalling Pathway

Cell line	Type	EBNA2 gene	EBNA2 expression	TAN-1_{wt}	TAN-1 truncated	HES1	Notch pathway
			Group 1a				
PBMC 1	Control	ND	–	9.8	–	–	Intact
PBMC 2	Control	ND	–	5.0	–	–	Intact
Daudi[a]	Burkitt lymphoma	–	–	–	–	0.3	Intact
EW-36	Undifferentiated lymphoma	–	–	4.9	–	0.1	Intact
			Group 1b				
BL135	Burkitt lymphoma	+	+/–	–	–	–	Intact
AS283	AIDS-B lymphoma	+	–	1.0	–	–	Intact
VAL	Large cell lymphoma	+	+/–	–	–	0.2	Intact
LY8	Large cell lymphoma	–	–	–	–	–	Intact
BL41	Burkitt lymphoma	–	–	–	–	–	Intact
			Group 2				
Raji	Burkitt lymphoma	+	++	–	–	4.0	CA [EBNA]
BC2	B-lymphoma	+	++	–	–	2.5	CA [EBNA]
			Group 3a				
SupT1	T-ALL	–	–	–	153.4[b]	3.7	CA
FRO	T-ALL	–	–	47.0	3.6	3.9	CA
			Group 3b				
MOLT4	T-ALL	–	–	3.58	–	2.7	CA
CEM	T-ALL	–	–	2.2	–	3.4	CA
KE37	T-ALL	–	–	1.9	–	2.8	CA

Table I summarises results relating to the expression of genes involved in the Notch pathway. The first row shows results about EBNA2 expression, they are obtained by RT-PCR and are qualitative values: – mark represents a lack of amplification; +/– represents low level of expression and ++ represents of high expression of EBNA2 gene. The figures provided represent ratios signal values in pixels of each gene/signal values in pixel of 18S RNA. Negative sign indicates that signals were not detectable.
EBNA, Epstein-Barr virus nuclear antigen-2; CA, constitutively activated; CA [EBNA], EBNA-mediated constitutive activation; ND, not determined.
[a]Daudi cell line is reported to be EBV infected (ATCC catalogue).
[b]The truncated TAN-1 form expressed in SupT1 is the result of a translocation found in T-ALLs and widely studied by Ellisen et al. [1991] causing the loss of the most of the extracellular domain of the protein with a consequent constitutive activation.

above specified number of cycles according to the following regime: denaturation at 94°C for 30 sec, annealing at the above specified degrees for 30 sec and extension at 72°C for the above specified time.

Analysis of EBNA2 Status

The cell lines that had been analysed by Northern blot were also tested for the presence of EBNA2 by PCR technique on genomic DNA and by RT-PCR on 1 μg of total RNA of each cell line previously treated with RNase free DNase (Promega Italia). RT conditions were as described above. The PCR conditions were as follows. RT-PCR was normalised by simultaneous amplification of each sample for both EBNA2 and the housekeeping gene *GAPDH*. Samples were electrophoresed on 2% agarose gel and for RT-PCR samples quantification of the signals was performed by using Kodak 1D image Analysis Software. The values obtained are ratios of pixels representing the whole area of the target gene signal/GAPDH signal.

Primers and PCR Conditions

TAN-1 (Southern blot) (Annealing 62°C, extension 1 min 30 sec)	Upper primer 5′-TTGCCTGTGCCCCGCCAAAT-3′ Lower primer 5′-CTCCCGCCGCTTCTTCTTGC-3′
TAN-1 (Northern blot) (Annealing 62°C, extension 1 min)	Upper primer 5′-GTCGCCCCCGCTCTGCTC-3′ Lower primer 5′-GCTTTGCTGCTGCTGGATGTT-3′
HES1 (Northern blot) (Annealing 54°C, extension 30 sec)	Upper primer 5′- TTGCTTTCCTCATTCCCAAC-3′ Lower primer 5′- AGGCGCAATCCAATATGAAC-3′
EBNA2 (Northern blot and RT-PCR) (Annealing 57°C, extension 30 sec)	Upper primer 5′- GCCAAACACCTCCAGTCCTA-3′ Lower primer 5′-ATACCAATCATCGGGGAAGAG-3′
GAPDH (Northern blot and RT-PCR) (Annealing 57°C, extension 30 sec)	Upper primer 5′-CCATGGAGAAGGCTGGGG-3′ Lower primer 5′-CAAAGTTGTCATGGATGACC-3′

RESULTS

Genomic Changes in TAN-1

The aim of the first part of the present work was to analyse the possibility of the presence of genomic mutations in *TAN-1* in haemopoietic neoplasms. We have analysed both myeloid leukaemia and T-acute lymphoblastic leukaemia primary samples. None of these showed genomic alterations. On account of the poor presence of lymphoma cells in the blood from patients we also analysed leukaemic and lymphoma cell lines for genomic alterations.

Digestion was performed with EcoRI and a restriction polymorphism was found for the enzyme. The restriction pattern of *TAN-1* gene (Fig. 1) showed two bands of approximately 15 and 8.2 kb except for LY12.3 cell line that showed a band of 3.7 kb instead of that of 8.2 kb. This anomalous band could correspond to chromosomal modification or quite simply to a polymorphism on LY12.3 genome in an EcoRI intronic restriction site, as there are no EcoRI restriction sites in the coding sequence of *TAN-1*. The genomic DNA was also digested with BamHI, in order to check for restriction polymorphism. All the samples tested showed an identical restriction pattern with two bands at approximately 6.1 and 4.2 kb (Fig. 1, lower panel). This has allowed us to conclude positively that neither LY12.3 cell line nor any of the other cell lines showed any evidence of functional polymorphism for TAN-1. No genomic alterations affecting TAN-1 were detected in any of the cell lines investigated.

Integrity of Notch Pathway

The absence of genomic abnormalities does not guarantee that the signalling pathway is functioning normally. Therefore the integrity of the *Notch* signalling pathway was investigated by examining the expression of *TAN-1*/*Notch1* and *HES1* in 14 leukaemia and lymphoma cell lines and control peripheral blood mononuclear cells (PBMC) derived from two healthy donors (Fig. 2). In order to provide a complete picture of the possible causes of the activation/inactivation of the Notch pathway, the cells were also

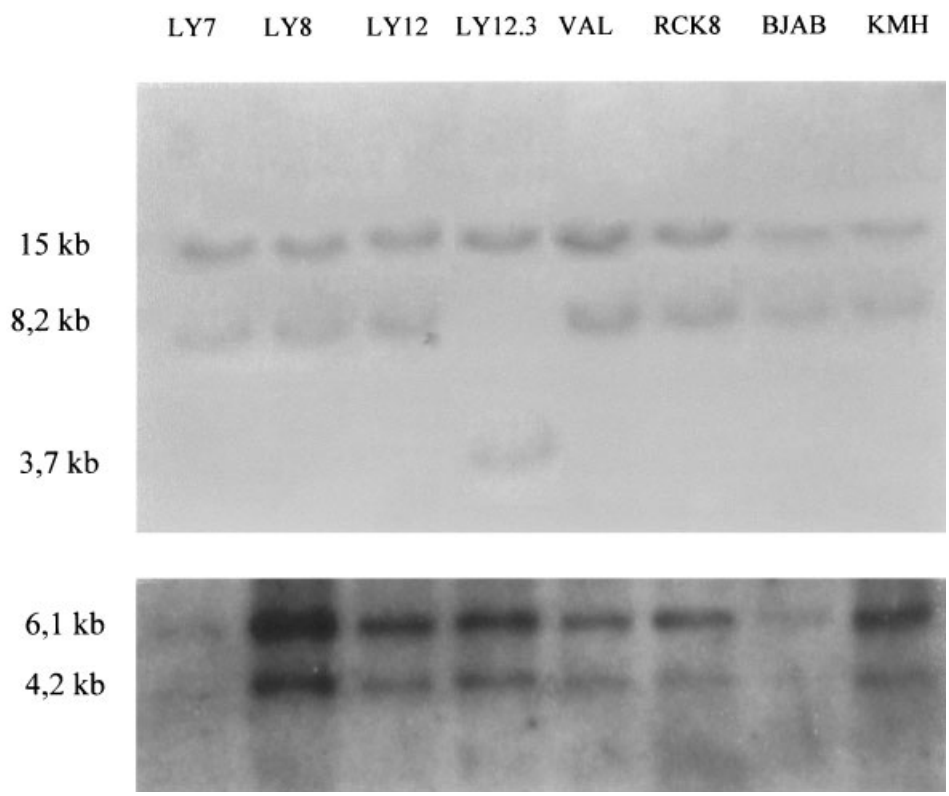

Fig. 1. Example of Southern blot analysis performed on DNA from lymphoma and leukaemia samples using probe for TAN-1. One polymorphism of LY12.3 cell line was detected in the EcoRI restriction pattern, not confirmed in the BamHI restriction (**lower panel**).

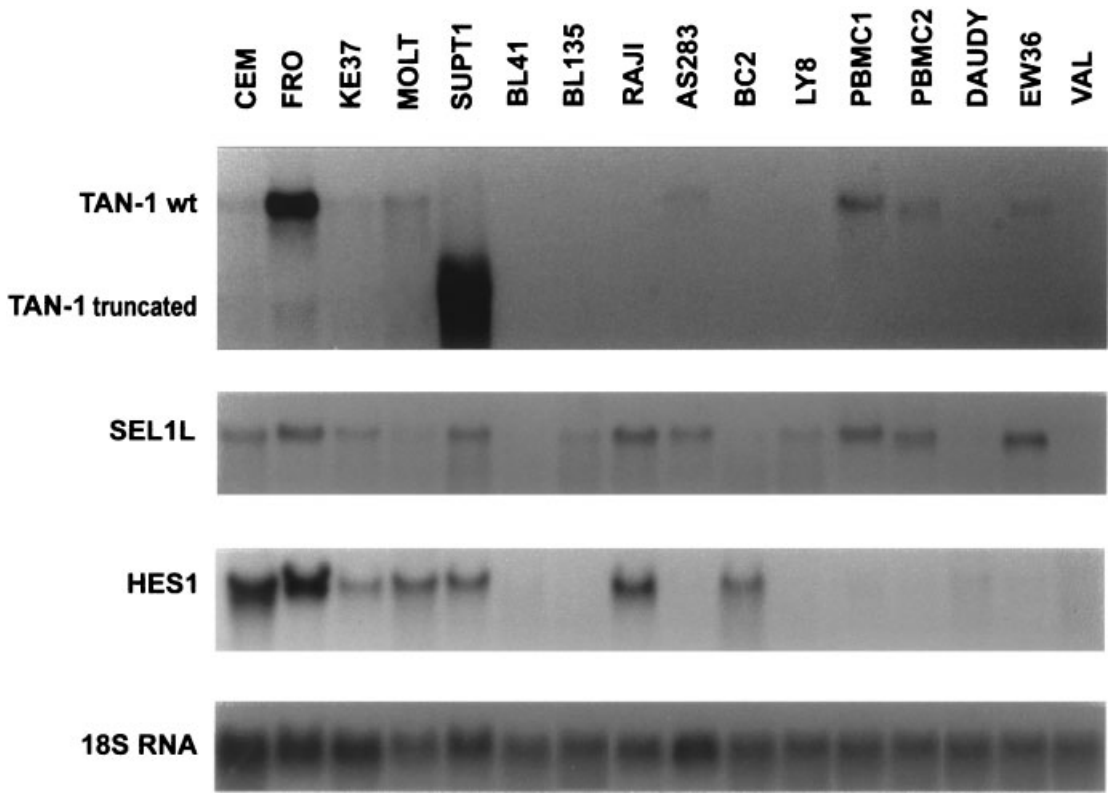

Fig. 2. Northern blots performed on lymphoma and leukaemia cell lines using probes for TAN-1, and HES1 and 18S RNA for normalisation. The putative negative regulator of Notch signalling, viz. SEL1L was also probed, but data not presented or discussed in the text.

tested for the presence in the cellular genome and the expression of the messenger of *EBNA2*, an EBV gene that mimics *TAN-1* (Fig. 3). The data are summarised in Table I.

From the present series of experiments, three groups can be defined in relation to the operation of Notch signalling. Group 1 constitutes cells like the control PBMC cells and lymphoma

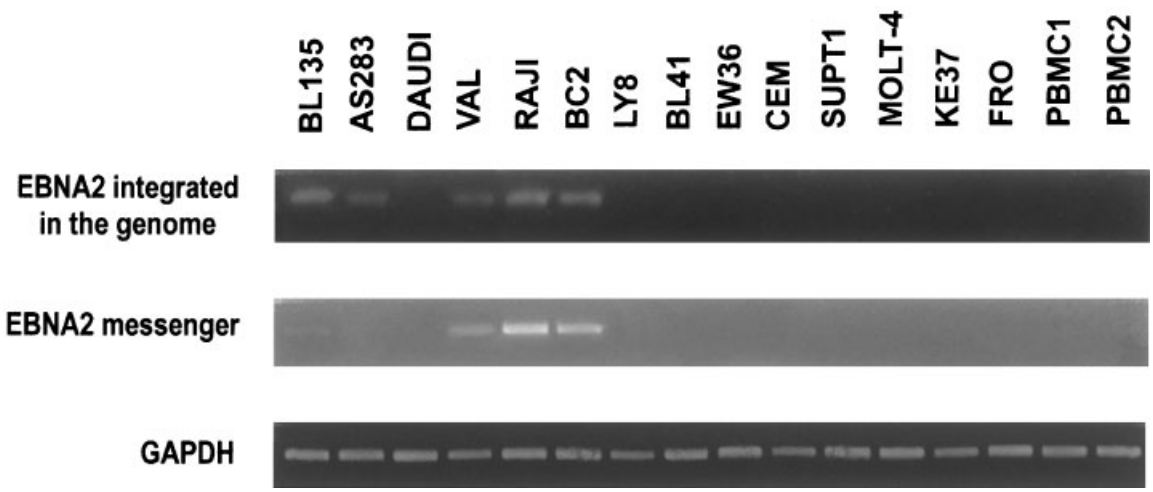

Fig. 3. Evaluation of EBNA2 status in lymphoma and leukaemia cell lines. Both insertion of EBNA2 in cell genome (by PCR on genomic DNA) and the level of expression of its messenger (by RT-PCR) are shown.

cell lines, where *HES1* is downregulated, which indicates that the Notch pathway is intact. This group is subdivided into Groups 1a and 1b. *TAN-1* is expressed in Group 1a, with the exception of Daudi cells. In Group 1b, only one expressed *TAN-1* at low levels. However, none of these showed *HES1* activation. In this group, three lymphomas were found to carry the *EBNA2* gene integrated in the genome, but only two of these expressed very low levels of EBNA2 messenger. None of the lymphomas was *HES1*-positive.

Group 2 contains only the B-lymphomas, Raji and BC2. Both were *TAN1* negative but both showed *HES1*. Both expressed *EBNA2* messenger at a high level, in contrast to group 1b lymphomas that showed no or low *EBNA2* expression or *HES1* activation. This indicates a close correlation between the expression of *EBNA2* and *HES1* activation.

Group 3 includes only the T-ALL cell lines MOLT4, CEM, SupT1, KE37 and FRO in which Notch signalling is constitutively active as indicated by *HES1* expression. We subdivided this into Groups 3a and 3b. The two Group 3a T-ALL cell lines FRO and SupT1 are characterised by the activation of Notch signalling caused by an alteration at TAN-1 level. It has been shown previously that SupT1 express a truncated constitutively active form of the receptor at high levels. On the other hand, FRO expressed the wild-type *TAN-1* at high levels and a truncated form at low levels. All three Group 3b cell lines show a constitutive activation of the pathway, even with low levels of *TAN-1*.

DISCUSSION

The Notch transmembrane protein plays an important role in the survival of precursor cells as well as in determining cell differentiation during haemopoiesis. The deregulation of Notch signalling leads to inhibition of differentiation, maintenance of the undifferentiated or precursor state and enhancement of cell proliferation. Although one can envisage how such abnormal states achieved through deregulated signalling can lead to the development of neoplasia, the functioning of the intracellular pathway and the factors that influence its normal functioning are still incompletely understood.

Notch signalling has been an area of intense interest in the wake of this gene being involved in consistent chromosomal translocations affecting its locus and characterised by proven tumorigenic ability. Our objective in the present study was to examine if any genetic abnormalities are associated with *TAN-1*, the human homologue of *Notch1*. We found no abnormalities in *TAN-1* gene in the myeloid leukaemia primary samples, T-ALL or lymphoma cells lines investigated here. One cannot exclude the possibility of mutations being present in other members of the Notch family. However, members of the family such as Notch 3, for instance, are comparatively less effective and poor activators of Notch signalling [Beatus et al., 2001]. It may be reasonable to suggest that the deregulation of Notch signalling was not a consequence of genomic changes in *TAN-1*. Hence we focused our attention on checking the integrity of the signalling cascade.

As shown in the Table I, the signalling cascade seems to be intact in Group 1 cell lines, where expression of the HES1 effector protein was low or undetected, even in the presence of high expression of TAN-1. This suggests transduction of the signal is still stringently regulated. In contrast, Group 1b cell lines showed no HES1 activation apparently due to the lack of activation of *TAN-1*. Interestingly, in this group, even *EBNA2*, seems to have produced no *HES1* activation. In the Group 2 lymphomas Raji and BC2, the constitutive activation of Notch signalling is attributable to EBNA2 gene and protein expression.

A most interesting finding relating to Group 3 is the apparent existence of two different forms of behaviour of T-ALLs with respect to the modality of the activation of Notch signalling. Group 3a presents activation due to alteration at the very high level of *TAN-1*. On the other hand, activation of the signalling pathway in Group 3b occurs even with *TAN-1* expression at comparatively low levels. Possibly, this suggests alterations in the receptor, different from those already described, or that alteration could be at a different level of the pathway, which could affect the same receptor or other members of the pathway.

Thus our data suggest that in leukaemia cells there could exist mechanisms activating signalling along the Notch pathway that are different from the ones involving a direct activation of Notch receptor. An elucidation of this potential distinction could help in understanding the pathogenesis of T-cell acute leukaemia.

It has been suggested on an empirical basis that Notch signalling is negatively regulated by *SEL1L* gene, which is the homologue of the *Caenorhabditis elegans* sel-1. It has been postulated that SEL1L interacts with the Notch receptor and downregulates it. It could be argued that abnormality in Notch signalling might reside in this putative negative regulator. Recent work by Chiaramonte et al. [2002] excludes this possibility. They investigated the cell lines employed in the present study but found no evidence of genomic alterations affecting SEL1L, nor an inverse relationship between SEL1L expression and the status of Notch signalling.

A particular case of T-ALL is represented by FRO cell lines that show a double alteration of the receptor TAN-1, a transcriptional upregulation of the wt form and the presence of a truncated protein. It remains to be seen as to which of the two anomalies cause the constitutive activation of signalling leading to upregulation of the effector HES1 and to further characterise the truncated form. Interestingly, the oncogenic potential of an over-expressed wild-type form of TAN-1 has not been reported to-date. Usually the truncated forms of TAN-1 are over-expressed, a situation different from that encountered here.

The present findings have important implications in the context of T-cell development and the pathogenesis of T-ALLs. New evidence shows a dichotomous, reciprocal role for Notch signalling in B and T cell development driving cells toward T lineage [Pui et al., 1999; Radtke et al., 1999]. This leads us to the final point for consideration that the most of the T-ALLs studied here show a constitutive activation of Notch signalling pathway. We postulate that the Notch pathway might play a key role in the development of T-ALLs category by preventing the normal differentiation of T-lymphocytes and favouring their proliferation, the two fundamental steps at the basis of tumorigenesis.

Although Notch and its ligand are upregulated in both leukaemias and lymphoma, in this study we have found marked differences between T-ALL and B-lymphomas in respect of notch signalling. In a majority of B-lymphoma cell lines the Notch pathway was intact and regulated and not invariably subverted by EBNA expression. In contract, in leukaemic cells the pathway was constitutively active and apparently deregulated. This is the first demonstration of a differential activation of the Notch pathway in these haematological malignancies.

In conclusion our findings are consistent with a constitutive Notch activation in T-ALLs and with an infrequent activation of this signalling pathway mediated by EBNA2 in B-lymphomas. They accord with the fact that B- and T-lymphocytes differ markedly as regards to interaction between Notch IC and CBF1/RBJ-Jκ. B cells show no Notch IC interaction with the transcription factor, but such association does occur in T-lymphocytes [Callahan et al., 2000]. The ability of Notch1 to participate in B cell gene regulation, reported by Strobl et al. [1997] seems to be restricted only to experimental conditions as Notch expression inhibits the ability of the cells to differentiate as B cells and our results confirm that Notch expression is extinguished in B-lymphomas.

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