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# Reciprocal Regulation of Notch and PI3K/Akt Signalling in T-ALL Cells In Vitro

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**Abstract** Notch signalling plays an important role in hematopoiesis and in the pathogenesis of T-ALL. Notch is known to interact with Ras and PTEN/PI3K (phosphoinositide-3 kinase)/Akt pathways. We investigated the interaction of Notch with these pathways and the possible reciprocal regulation of these signalling systems in T-ALL cells in vitro. Our analyses indicate that the PI3K/Akt pathway is constitutively active in the four T-ALL cell lines tested. Akt phosphorylation was not altered by the sequestration of growth factors, that is, Akt activation seems to be less dependent on but not completely independent of growth factors, possibly being not subject to negative feedback regulation. PTEN expression was not detected in 3/4 cell lines tested, suggesting the loss of PTEN-mediated Akt activation. Inhibition of the PI3K/Akt pathway arrests growth and enhances apoptosis, but with no modulation of expression of Bax-α and Bcl-2 proteins. We analysed the relationship between Notch-1 and the PI3K/Akt signalling and show that inhibition of the Akt pathway changes Notch expression; Notch-1 protein decreased in all the cell lines upon treatment with the inhibitor. Our studies strongly suggest that Notch signalling interacts with PI3K/Akt signalling and further that this occurs in the absence of PTEN expression. The consequences of this to the signalling outcome are yet unclear, but we have uncovered a significant inverse relationship between Notch and PI3K/Akt pathway, which leads us to postulate the operation of a reciprocal regulatory loop between Notch and Ras-PI3K/Akt in the pathogenesis of T-ALL. J. Cell. Biochem. 103: 1405–1412, 2008.


**Key words:** apoptosis; Bax; Bcl2; cell proliferation; Notch signalling; PI3K/Akt; PTEN; T-ALL

The Notch signalling pathway, a mechanism of signalling highly conserved in evolution, plays an important role in the development and differentiation of metazoans. Notch signalling is intricately involved in determining a broad spectrum of cell fates and regulating developmental processes. Thus it regulates neurogenesis, myogenesis, vasculogenesis, hematopoiesis, skin development and other aspects of organogenesis. Deregulation of Notch signalling by mutations of Notch receptors has been associated with the pathogenesis of T-cell leukaemia and developmental disorders such as cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL) and Alagille syndrome. Notch receptor activation by ligands leads to proteolytic cleavages resulting in the release of extracellular domain, and then to the release of the intracellular domain (ICN). The ICN possesses nuclear localisation signals that cause its translocation into the nucleus where it binds to a transcriptional regulator and activates a number of down stream target genes [reviewed by Chiaramonte et al., 2005].

Notch signalling plays a role in hematopoiesis [Milner and Bigas, 1999; Radtke et al., 2004]. Its role in tumorigenesis was identified with the involvement of Notch-1 in chromosomal translocation t(7;9)(q34;q34.3) that results in the fusion of 3′ portion of Notch-1 on chromosome 9

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to the Jβ joining region of the T-cell antigen receptor-β on chromosome 7. This genetic rearrangement generates a truncated constitutively active Notch-1 receptor, which is constitutively over expressed and is oncogenic [Pear et al., 1996]. Notch might be involved in the pathogenesis of T-ALL even in the absence of the translocation but with the presence of activating mutations in >50% of human T-ALL [Weng et al., 2004].

The classical Notch signalling pathway interacts with other signalling systems, such as the Ras pathway that transduces growth factor signals. The PI3K/Akt pathway has been recognised as an important signalling route for a variety of growth factors such as the Erb family members, PDGF, TGFα and angiogenic agents. The Ras-mediated information flow in cellular transformation can take the extracellular signal-regulated kinase (ERK)/mitogen-activated protein (MAP) kinase pathway or the PI3K route. There is much evidence that Notch interacts with Ras pathway, and also with the PI3K/Akt pathway that is regulated by the tumour suppressor gene PTEN. The findings of Fitzgerald et al. [2000] that Notch-4 mediated oncogenic transformation required the down stream ERK/MAP kinase and the PI3K pathways of Ras have prompted the present study into the role of Notch-1 in T-ALL. Here we have attempted to elucidate the interaction of Notch with the PI3K/Akt pathway and show for the first time the possible reciprocal regulation of these signalling systems in T-ALL cells in vitro.

## MATERIALS AND METHODS

### Cell Lines and Their Maintenance In Vitro

Human T-ALL cell lines Jurkat, FRO, SUP-T1 and CEM were cultured in RPMI 1640 (Euroclone, Devon, UK) supplemented with 10% foetal calf serum (FCS, Sigma), 2 mM L-glutamine (Euroclone) and 10 mM penicillin/streptomycin (Sigma). Medium for Jurkat and CEM was also supplemented with 10 mM Hepes (Sigma), 1 mM sodium pyruvate and 12 mM glucose. Cells were grown at 37°C in 5% $CO_2$.

### PBMC Separation

Peripheral blood mononuclear cells (PBMC) from healthy donors were separated on Ficoll-Hypaque gradient.

### Growth of Cells in Serum-Free Media

For some experimental work cells from the four cell lines were collected, washed with PBS and resuspended in serum-free media containing 2 mM L-glutamine. Then cells were grown for 24 h at 37°C under 5% $CO_2$ atmosphere.

### PI3K Inhibition

Cells were seeded at $0.3 \times 10^6$/ml and treated with PI3K inhibitor when they had reached the concentration of $0.5 \times 10^6$/ml. The PI3K inhibitor LY294002 (Cell Signalling Technology) was dissolved in dimethyl sulphoxide (DMSO) and added at a working concentration of 50 μM. Cells were grown in the presence of the inhibitor for 24 or 48 h. Control cells were grown in the same way by adding the same amount of DMSO (0.1%) as in treated cells.

### Cell Proliferation Assay (MTT Test)

The proliferation inhibition rate of LY294002 was determined after 24 and 48 h of treatment by using the MTT [3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide] uptake method. Briefly, after the treatment 100 μl aliquots of cell suspension ($0.5 \times 10^6$ cells/ml) of each of the four cell lines were seeded in triplicate in 96-well plates. The same was done for control cells. After 48 h of culture, 10 μl of MTT (6 mg/ml, SIGMA) was added to each well. The plates were incubated for 4 h at 37°C under 5% $CO_2$. Then the medium was removed from each well with a syringe needle and the formazan crystals formed in the viable cells were resuspended in 100 μl of isopropanol + HCl 0.025N. The plates were read at 550–570 nm (L1) and 620–650 nm (L2) as reference on scanning multiwell Spectrophotometer OD = L1−L2. Percentage of cell survival is expressed as: (absorbance of treated wells/absorbance of control wells) × 100.

### Assay of Apoptosis

Cells undergoing apoptosis were identified by Annexin-V and propidium iodide (PI) staining (Medical and Biological Laboratories MBL) according to the manufacturer's instructions. Phosphatidylserine, which is normally located on the cytoplasmic side of cell membrane, is translocated to the cell surface upon induction of apoptosis. Annexin V binds to phosphatidylserine and is used as a probe to identify the early stages of apoptosis. PI, which does not enter cells with intact membranes, is used to

distinguish between early apoptotic cells (Annexin V-positive) and late apoptotic or necrotic cells (Annexin V-PI-double positive). To determine the proportion of apoptotic cells after 24 and 48 h of incubation, $2 \times 10^5$ cells were collected by centrifugation and resuspended in 500 μl of 1× Annexin V binding buffer (10 mM HEPES/NaOH pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$). One microlitre of Annexin V-FITC and 1 μl of PI (MBL) was then added and cells were incubated at room temperature for 5 min in the dark. Samples were then centrifuged and resuspended in 15 μl of PBS. The suspension was added on to a glass slide and covered with a glass cover slip. Apoptotic cells were visualised by fluorescent microscopy.

### Protein Extraction and Western Blot Analysis

Cells ($4 \times 10^6$) were harvested, washed with phosphate buffered saline (PBS) and then lysed in 40 μl of RIPA (radioimmuno-precipitation assay) buffer containing a mixture of protease inhibitors (50 mM Tris-HCl 1 M PH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% triton X-100, 0.1% SDS, 1.0% sodium deoxycholate, 2 mM sodium fluoride, 1 mM sodium orthovanadate, 2 μg/ml aprotinin, 2 μg/ml leupeptin, 100 μg/ml phenylmethylsulfonyl fluoride, 1 μg/ml pepstatin A and 50 μg/ml tosyl-lysine chloromethyl ketone). After incubation on ice for 50 min, the lysate was clarified by centrifugation for 10 min at 4°C. Protein concentration of the cell lysate was determined using the Bradford assay (Bio-Rad Laboratories). Protein samples (40–80 μg) were loaded onto and run on SDS-polyacrylamide gels, transferred to a nitrocellulose membrane (Hybond-ECL, Amersham Bioscience), and blocked with 5% powdered milk in TBST (50 mM Tris, pH 7.5, 150 mM NaCl, 0.01% Tween-20). The membrane was then incubated with primary antibody diluted in 5% powder milk in TBST, washed extensively and incubated with HRP-conjugated species-specific secondary antibodies (Santa-Cruz Biotechnology). Proteins were visualised with ECL reagents (Amersham Biosciences) according to the manufacturer's instructions. Uniform loading of proteins was confirmed by Ponceau S staining.

The antibody against Notch-1 (C-20, Santa-Cruz biotechnology) recognises an epitope mapping at the C terminus of the ICN of the protein. Antibodies against Bcl2, Bax-α and β-actin were obtained from SIGMA and the antibodies against pAkt/PKB, total Akt/PKB and PTEN were from Cell Signalling Technologies.

### RT-PCR

Total RNA was extracted from $3 \times 10^6$ cells/sample (Chomczynski/Sacchi method) and 1 μg RNA was retro-transcribed in 20 μl by M-MLV reverse transcriptase (Invitrogen Life Technologies) according to manufacturer's guidelines. PCR was performed using 2 μl of cDNA, 1× PCR buffer, 1.5 mM $MgCl_2$, 200 μM dNTPs, 1 μM each primer, 0.25 U AmpliTaq DNA polymerase (Applied Biosystems). The following primers were used:

- GAPDH fwd CCATGGAGAAGGCTGGGG, rev CAAAGTTGTCATGGATGACC,
- Notch fwd CCCACTCATTCTGGTTGTCG, rev CGCCTTTGTGCTTCTGTTCT,
- Hes1 fwd ACGACACCGGATAAACCAAA, rev CGGAGGTGCTTCACTGTCAT,
- pTα fwd CTGGATGCCTTCACCTATGG, rev CAGGTCCTGGCTGTAGAAGC,
- Deltex fwd CTTCCCTGATACCCAGACCA, rev CGTGCCGATAGTGAAGATGA.

## RESULTS

The experiments described here were aimed at determining whether Notch signalling in T-ALL uses either of Ras or the PI3K/Akt pathway, and whether the latter might be subject to co-regulation by Notch and other apoptotic signalling systems.

### PI3K/Akt Pathway in T-ALL

We investigated the effects of the PI3K specific inhibitor LY294002 on the growth and survival of T-ALL cell lines to demonstrate the involvement of PI3K/Akt activation in these cell lines. LY294002 (50 μM) treatment of all the four cell lines resulted in 60–80% inhibition of cell growth (Fig. 1) after 48 h. This loss of cell growth appears to be due to enhanced apoptosis; all the cell lines employed here showed increased apoptosis as result of LY294002 treatment (Fig. 2). A Western blot analysis showed that no modulation of expression of the apoptosis related Bax-α and Bcl-2 genes (Fig. 3).

### Activation of Akt Signalling and Its Negative Feedback Regulation

We excluded the interference by growth factors in Akt activation by demonstrating that

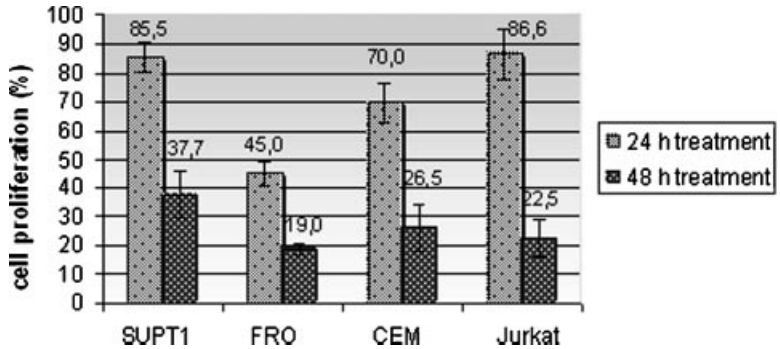


**Fig. 1.** Cell proliferation after LY294002 treatment compared to control cells in which proliferation is considered 100%.

the Akt pathway was constitutively activated in the cell lines employed. We examined Akt/PKB phosphorylation levels after 24 h of growing in serum-free media. As shown in Figure 4, Akt pathway does not seem to be inhibited; indeed Akt phosphorylation showed no decrease whilst total Akt was unchanged (data not shown). This shows Akt activation is less dependent on but not completely independent of growth factor; indeed after 48 h, phosphorylation decreased but cells suffered, possibly being not subject to negative feedback regulation.

### Loss of PTEN and Akt Activation

The suppressor gene PTEN has been shown to suppress cell proliferation and tumour cell population expansion by inhibiting Akt kinase, which possesses marked antiapoptosis properties. We checked the PTEN expression status and found that 3/4 cell lines showed no detectable PTEN protein, whilst one (SUP-1) expressed the protein at a low level, as compared with control PMBC cells (Fig. 5). This suggests the possibility that the activation of Akt pathway could be a result of the loss of Akt suppression normally exerted by PTEN.

### Modulation of Notch in Parallel With Akt Activation

In order to determine the relationship between Notch signalling and the activation of the Akt pathway, we investigated the changes in Notch expression by inhibiting the Akt pathway using PI3K inhibitor. We found decreased expression of Notch-1 protein in all

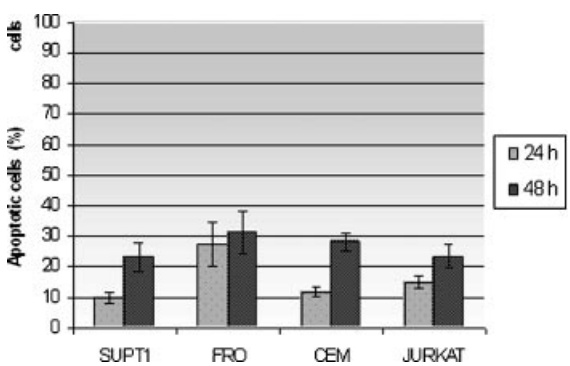


**Fig. 2.** Percentage of apoptotic cells after 24 and 48 h of treatment with LY294002. Apoptosis in the control cells was always 0–2%. We have subtracted control value from the actual apoptosis.

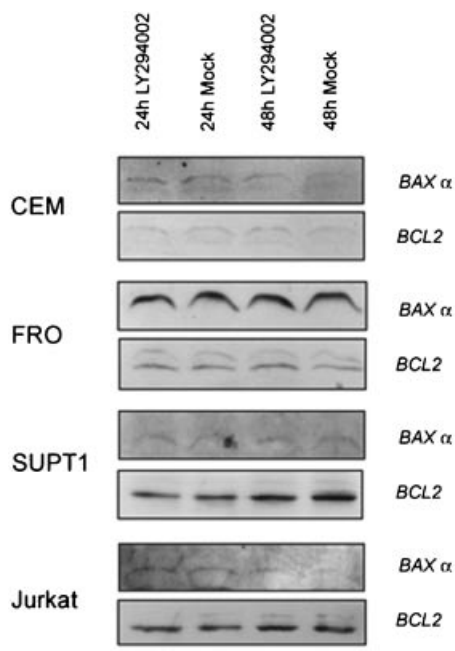


**Fig. 3.** BAX-α and BCL2 protein levels are not modulated by the treatment with LY294002. The picture is a representative of three independent experiments. *BAX* α in CEM, SUPT1 and Jurkat was hardly detectable by western blot technique. Mock: control cells.

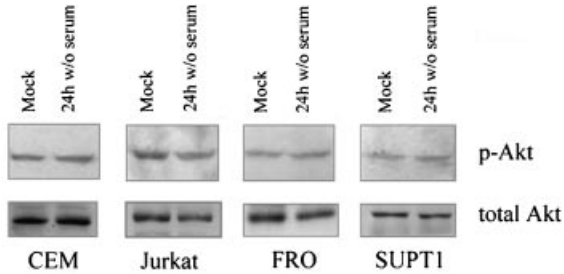


**Fig. 4.** Akt/PKB phosphorylation levels and total Akt levels after 24 h of growth in serum-free media. Mock: control cells grown in complete medium; 24 h w/o serum: cells grown in medium without serum for 24 h. β-Actin was used to normalise (data not shown).

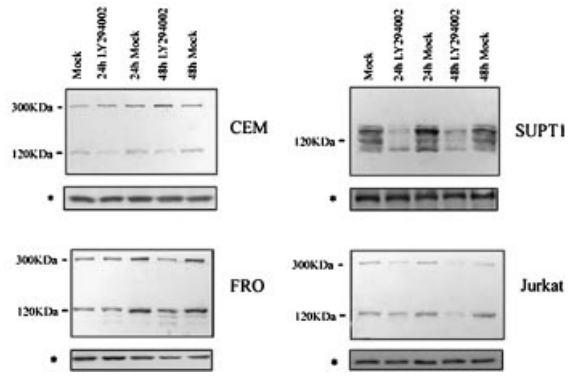


**Fig. 6.** Modulation of Notch1 after 24 and 48 h of treatment with LY294002. Mock: control cells at time 0; 24h mock and 48h mock: control cells at 24 and 48 h. β-Actin (marked by asterisk) was used to normalise.

the cell lines upon treatment with the inhibitor (Fig. 6). However, no changes were found in the Notch 1 gene expression (Fig. 7).

We ascertained the integrity of Notch signalling by looking at the modulation of expression of the down stream target genes Hes1 and pre-Tα and also for Deltex. Deltex is a component of the Notch signalling pathway and is believed to interact genetically with Notch and others genes in the pathway in a manner consistent with it acting as a positive regulator of Notch. The modulation of none of these genes followed a definable pattern as shown in Figure 8 and summarised in Table I.

## DISCUSSION

The apparent deregulation of Notch signalling in the pathogenesis of leukaemias and lymphomas has stimulated investigations of the mechanisms by which Notch ligand binding to the receptors conveys the signal leading to phenotypic changes. According to Weng et al. [2004] more than 50% of human T-ALLs, including tumours from all major molecular oncogenic subtypes, have activating mutations in the extracellular heterodimerisation domain and/or the C-terminal PEST domain of Notch-1. The presence of the ligand is not required for Notch activation. Many oncogenes have been suggested as being potentially involved in Notch signalling mechanism. Interaction of Notch signalling with cell cycle control genes is apparent from the demonstration that the introduction of activated Notch in SCLC cells leads to cell cycle arrest and this is accompanied by increase in the expression of cdk inhibitors $p21^{waf1}$ and $p27^{kip1}$ [Sriuranpong et al., 2001]. Ronchini and Capobianco [2001] also found

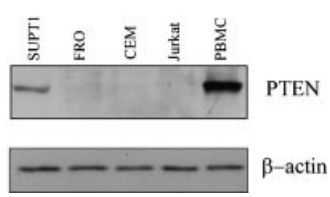


**Fig. 5.** PTEN protein expression in the four T-ALL cell lines and in control PBMC. PBMC: peripheral blood mononuclear cells. β-Actin was used to normalise.

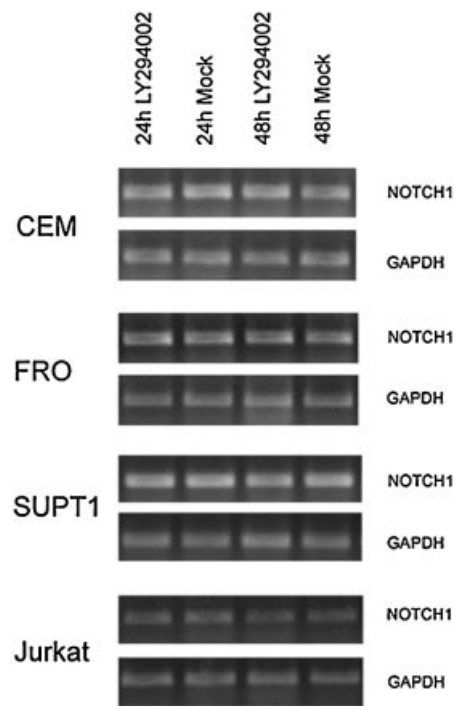


**Fig. 7.** Notch1 gene expression is not modulated after the treatment with LY294002. RT-PCR shown is representative of three independent experiments. The housekeeping gene GAPDH has been used for normalisation.

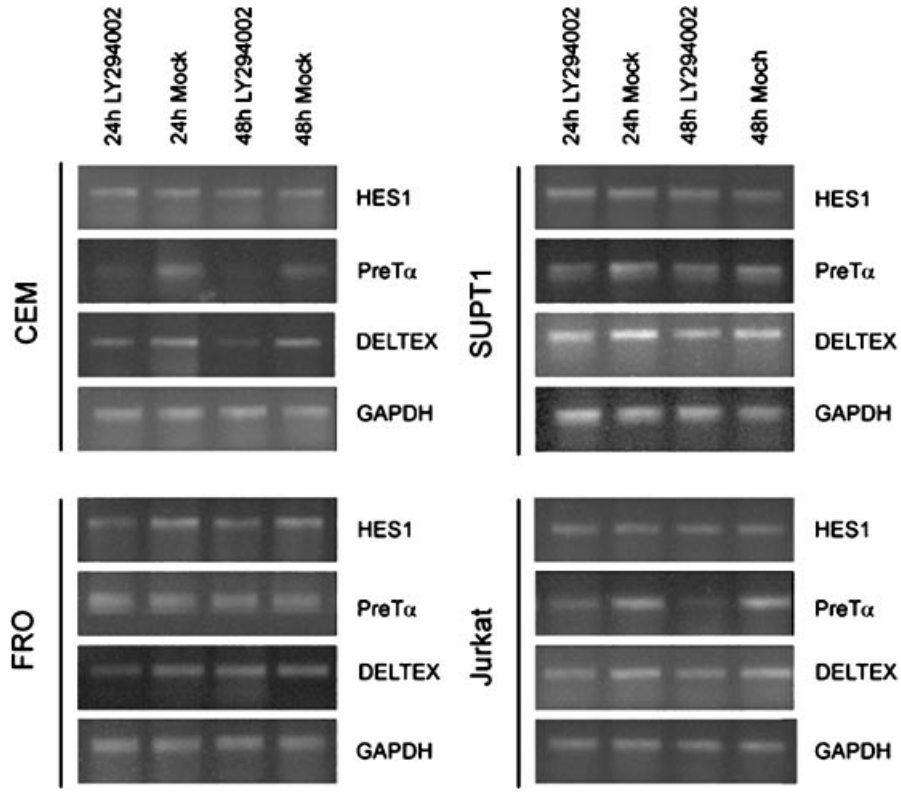


**Fig. 8.** HES1, PreTα and DELTEX gene expressions after the treatment with LY294002. RT-PCR analyses were performed on three independent experiments. The housekeeping gene GAPDH has been used for normalisation.

**TABLE I. The Modulation of Genes Down Stream of the Notch Receptor Indicative of the Integrity of the Pathway, as Assayed by RT-PCR (See Also Fig. 8)**

| | 24 h | 48 h |
|---|---|---|
| CEM | | |
| HES1 | = | = |
| pTα | ↓↓ | ↓↓ |
| DELTEX | = | ↓ |
| FRO | | |
| HES1 | ↓ | ↓ |
| pTα | = | = |
| DELTEX | ↓ | = |
| SUPT-1 | | |
| HES1 | = | = |
| pTα | ↓ | ↓ |
| DELTEX | ↓ | ↓ |
| Jurkat | | |
| HES1 | = | = |
| pTα | ↓ | ↓↓ |
| DELTEX | ↓ | ↓ |

↓: light/weak decrease, ↓↓: strong decrease, =: invariable.
Hes1 and pre-Tα and also Deltex are down stream target genes of Notch. Deltex is a component of the Notch signalling pathway and is believed to interact genetically with Notch and others genes in the pathway in a manner consistent with it acting as a positive regulator of Notch.

increased and constitutively enhanced expression of cyclin D1 in cells containing activated Notch ICN. Furthermore, the p53 family proteins, p63 and p73 are capable of up regulating the expression of the two Notch ligands Jagged 1 and Jagged 2, [Sasaki et al., 2002]. Activated forms of Notch-1 complement the functions of HPV E7 and E8 in cell transformation by activating the PI3K/Akt pathway [Rangarajan et al., 2001]. HPV oncoproteins do sequester and abrogate the cell cycle regulatory function of p53 function and in this way they could be influencing the outcome.

There are clear indications that Notch signalling might recruit other pathways such as Ras pathway, along the ERK/MAP kinase and the PI3 kinase lines, but Notch-mediated transformation does not use src-like Lck and Fyn kinases, nor does it depend upon signals from protein kinases A and C (PKA, PKC) [Fitzgerald et al., 2000]. Fitzgerald et al. investigated the interaction between Notch-4 and Ras and the requirement of Ras in Notch mediated

oncogenesis. This prompted us to investigate the possible interaction between the two pathways using a different model in which, Notch-1, not Notch-4, was clearly deregulated. This implicates Ras as a collaborating component with Notch receptor signalling. Weijzen et al. [2002] have reported that oncogenic Ras can activate the Notch pathway and indeed it increases levels and activity of the intracellular form of wild type Notch-1, and also up regulates the expression of Notch ligand Delta-1 and also presenilin-1, which is involved in Notch processing.

There is a large body of evidence that attributes a major role for PI3-kinase in oncogenic transformation and cancer progression. PI3K activation generates phosphatidylinositol phosphates, $PIP_2$ and $PIP_3$. The tumour suppressor PTEN, which possesses a lipid phosphatase activity, negatively regulates PI3K activity and reduces Akt activity. Mutation and loss of PTEN expression results in uncontrolled activation of PI3K/Akt in a number of murine and human multiple myeloma cell lines [Hyun et al., 2000] where the constitutive activation of Akt displayed a clear antiapoptotic effect.

Our analyses indicate that the PI3-kinase pathway is constitutively active in the four T-ALL cell lines tested and that it plays a critical role in the growth and survival of T-cells. Indeed, inhibition of PI3K by LY294002 and the consequent incapacitation of the PI3K/Akt pathway markedly reduced cell growth with attendant increases of apoptotic loss of cells. The enhancement of apoptosis was not accompanied by changes in the expression of Bax-$\alpha$ or Bcl2 protein expression. This can be interpreted as excluding the operation of p53-mediated pathway in the apoptotic process. Although we have not observed any change in Bax-$\alpha$ and Bcl2 protein levels it seems possible that these proteins could be modulated by other means, such changes in their intracellular localisation that can modulate their activity.

We are especially mindful in this context that with PI3K inhibition the inhibition of Bclx-L has been previously reported in 2 of the 4 cell lines which we have employed here [Uddin et al., 2004]. The absence in bax and Bcl2 modulation does necessarily mean that they do not have a role in apoptosis; it is possible that their affinity for the mitochondrial membrane can change without their expression being modulated. On the other hand, SUPT-1 cells show significantly high levels of proliferation. So an alternative thesis would be to entertain the possibility of the presence of PTEN mutations in these cells, since PI3K inhibition does suppress Akt pathway in the presence of PTEN loss or mutation.

In three of the cell lines investigated the loss of PTEN is probably responsible for the deregulation of Akt activation and the maintenance of an antiapoptotic background. However, Akt phosphorylation reflecting regulation/turnover can occur without ligand stimulation, that is, without the stimulation of ligands by growth factors [Noro et al., 2006] and also independently of PTEN [Schick et al., 2006]. Equally, other events such as mutation of the PI3KCA in the PI3K/AKT pathway would result in constitutive activation of Akt. Compatible with this notion, is the finding that Akt is highly phosphorylated when mutations occur in PIK3CA [Bertelsen et al., 2006; Kozaki et al., 2006]. This could explain the activation of Akt in SUPT1 cells.

Since the PI3K-PKB/Akt pathway is a key effector of Ras, it could be suggested that Notch signalling might require active co-operation from the Ras pathway for the maintenance of the neoplastic phenotype, also in our T-ALL cell lines. So we decided to analyse the relationship between Notch-1 and the PI3-Kinase pathway in the four T-ALL cell lines.

In order to take this investigation further, we examined the effects of LY294002 on Notch expression. As described earlier, this inhibitor enhanced apoptosis, but the p53-regulated Bax-$\alpha$ and bcl-2 genes were unaffected. Treatment with the inhibitor also reduced Notch protein levels in all four cell lines tested, but had had no effect on gene expression. This suggests the involvement of new elements, which might be relevant in both contexts of Notch and PI3K/Akt signalling pathways. In a recent paper, Chappell et al. [2005] showed increased expression of PTEN in the presence of constitutively active Notch-1; but this does not appear to be the case here. However, here we find that the Akt inhibitor down regulates Notch and increases apoptosis. Thus overall, our studies do support the link-up of Notch signalling with hyperactivation of PI3K/Akt signalling in these T-ALL cells and further that this occurs in the absence of PTEN expression, except in SUPT1 cells. We found that serum sequestration did

not inhibit Akt; indeed Akt phosphorylation decreased and there were no changes in total Akt levels. This has suggested that Akt activation is less dependent on but not totally independent of growth factors, possibly being not subject negative feedback regulation.

## CONCLUDING REMARKS

Here we have examined the role of Akt in the pathogenesis of T-ALL. Akt is constitutively activated by phosphorylation. Akt phosphorylation was not altered by sequestration of growth factors, that is, Akt activation appears to be less dependent on but not completely independent of growth factors, possibly being not subject to negative feedback regulation. We show that PI3K/Akt constitutive activation has an important role in inhibition of apoptosis in our T-ALL cell lines. Our studies strongly suggest that Notch signalling interacts with PI3/Akt signalling in these T-ALL cells and further that this occurs in the absence of PTEN expression. The significance in terms of signalling outcome is yet unclear. However, we have uncovered an inverse relationship between Notch and PI3K/Akt pathway, which leads us to postulate the operation of a reciprocal regulatory loop between Notch and Ras in the pathogenesis of T-ALL.

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