Loss of nuclear expression of the p33\textsuperscript{ING1b} inhibitor of growth protein in childhood acute lymphoblastic leukaemia

G S Nouman, J J Anderson, K M Wood, J Lunec, A G Hall, M M Reid, B Angus

Background/Aims: p33\textsuperscript{ING1b} is a tumour suppressor protein involved in growth control and apoptosis. Suppression of p33\textsuperscript{ING1b} expression is associated with the loss of cellular growth control and immortalisation, whereas its overexpression causes cell cycle arrest. Moreover, normal p33\textsuperscript{ING1b} expression is essential for optimal function of p53. Acute lymphoblastic leukaemia (ALL) is the most common malignancy of childhood, accounting for one third of all childhood malignancies. A variety of cytogenetic abnormalities have been described but there is no single abnormality common to all cases. Deregulation of the TP53 pathway is a common genetic abnormality in human malignancies. However, TP53 mutations are uncommon in ALL. It is possible that alternative mechanisms of regulation of the TP53 apoptosis pathway, such as modulation of p33\textsuperscript{ING1b} expression, may be important in ALL. The aim of this study was to assess the expression of p33\textsuperscript{ING1b} in childhood ALL.

Methods: One hundred and forty five patients with childhood ALL were investigated in this immunohistochemical study of the expression of p33\textsuperscript{ING1b}.

Results: Loss of nuclear expression of p33\textsuperscript{ING1b} was seen in 78% of cases. This was associated with increased cytoplasmic expression of the protein. Kaplan Meier survival analysis demonstrated a trend towards a better prognosis for patients with tumours that had lost nuclear p33\textsuperscript{ING1b}.

Conclusion: These results suggest that the loss of nuclear p33\textsuperscript{ING1b} expression may be an important molecular event in the pathogenesis of childhood ALL.

Garkavtsev et al were the first to describe the inhibitor of growth 1 (ING1) gene in 1996. Since then, it has been mapped to a locus on the long arm of chromosome 13 (13q33–34).\textsuperscript{1} The ING1 gene consists of three exons that encode a series of spliced mRNAs,\textsuperscript{1} which may be translated to produce three proteins: p47\textsuperscript{ING1a}, p33\textsuperscript{ING1b}, and p24\textsuperscript{ING1c}.\textsuperscript{2–4} The most widely expressed of these proteins appears to be p33\textsuperscript{ING1b}\textsuperscript{1,5–7}.

Functional studies have shown that pro ducts of the ING1 gene are involved in the restriction of cell growth and proliferation, apoptosis, anchorage dependence, cellular senescence, the maintenance of genomic stability, and the modulation of cell cycle checkpoints.\textsuperscript{8–10} Suppression of p33\textsuperscript{ING1b} is associated with the loss of cellular growth control and immortalisation, whereas its overexpression arrests cells in the G0/G1 phase of the cell cycle.

Co-immunoprecipitation studies have indicated that ING1 gene protein products interact with the TP53 tumour suppressor gene protein product p53, whereas co-transfection studies demonstrate that ING1 has the ability to modulate TP53 transactivation of the cyclin dependent kinase inhibitor WAF1.\textsuperscript{11} Extension of these preliminary findings suggested that the association of competent protein forms of each member of the ING1–TP53 complex is essential for optimum inhibition of cell growth and transactivation by TP53.\textsuperscript{12}

We have shown by means of immunohistochemistry that p33\textsuperscript{ING1b} is expressed in the nuclei of cells in almost all human tissue types, with cytoplasmic expression in a more restricted range of tissues. Recent studies have reported reduced ING1 mRNA expression in lymphoid malignancies, gastrointestinal tumours, and breast carcinomas.\textsuperscript{13–15} In contrast, mutations in ING1 appear to be extremely rare in breast carcinoma, colorectal carcinoma, head and neck tumours, squamous carcinoma, and other tumours.\textsuperscript{16–22}

“Loss of p21\textsuperscript{WAF1} might lead to failure of cellular growth suppression, possibly through deregulation of the retinoblastoma gene”

Acute lymphoblastic leukaemia (ALL) is the most common malignancy of childhood, accounting for one third of all childhood malignancies.\textsuperscript{23} Approximately 2500 new cases of ALL are diagnosed each year in the USA.\textsuperscript{24} ALL occurs more frequently in individuals less than 15 years of age, and it is more common in males and white individuals.\textsuperscript{25} ALL is composed of different neoplastic subtypes of immature precursor lymphoblasts. For what was once a fatal disease, remission is now induced in 95% of cases of ALL and the five year survival rate is 70%.\textsuperscript{26–28} This incredible improvement in response is mainly the result of advances in multi adjuvant chemotherapy and supportive care.

About 90% of patients have a chromosomal abnormality in the leukemic cells, such as hyperdiploidy (trisomy 12), deletions (13q), gene rearrangements (TAL1), and translocations (t12;21, t9;22, t9;14, and t4;11). A range of cell cycle inhibitors and regulators have been studied for their potential role in the development and progression of ALL, such as members of the INK family gene (p16\textsuperscript{INK4a}, p15\textsuperscript{INK4b}), the CIP/KIP family member p21\textsuperscript{WAF1}, the retinoblastoma gene (RB), and the tumour suppressor p53.\textsuperscript{29–33} Loss of p16\textsuperscript{INK4a} and p15\textsuperscript{INK4b} expression are frequent in childhood ALL and result in deactivation of the RB gene. Moreover, loss of p21\textsuperscript{WAF1} might lead to failure

Abbreviations: ALL, acute lymphoblastic leukaemia; B-ALL, B cell acute lymphoblastic leukaemia; ING1, inhibitor of growth 1; RB, retinoblastoma gene; T-ALL, T cell acute lymphoblastic leukaemia; TBS, Tris buffered saline; WBC, white blood cell
of cellular growth suppression, possibly through deregulation of the RB gene.

Because TP53 mutations are uncommon in ALL at diagnosis,14 and deletion of chromosome 13q is common in ALL, it is possible that deregulation of the p53 apoptosis pathway by modulation of p33ING1b expression may be an important molecular mechanism in ALL. Therefore, we undertook our study to investigate the expression of p33ING1b in 145 patients with childhood ALL.

MATERIALS AND METHODS

Patients and tissues studied

The patients studied comprise a series of 145 cases of ALL diagnosed at the Royal Victoria Infirmary, Newcastle upon Tyne, UK, between 1985 and 1997. Bone marrow trephine biopsies were taken at diagnosis. Follow up data, including age, sex, presenting white blood cell (WBC) count and survival data were available for all patients. All patients were entered into clinical trials established by the Medical Research Council to assess the relative qualities of different treatment regimens for childhood ALL. These were UKALL X (n = 61), UKALL XI (n = 24), and UKALL XI’92 (n = 60). All patients received standard induction treatment containing daunorubicin and vincristine, with supplementary methotrexate in intensification blocks.15 Normal human bone marrow trephine specimens and normal human thymus were used as positive controls for immunohistochemistry.

Monoclonal antibodies used

Monoclonal antibodies used in our study were anti-p53 (NCL-DO7, Novocastra, Newcastle, UK) and anti-p33ING1b (GN1), which was generated in our laboratories.16 The GN1 monoclonal antibody was generated using a full length p33ING1b recombinant protein as an immunogen in the production and selection of specific monoclonal antibody secreting hybridoma cell lines.

Statistical analysis

To assess the relation between p33ING1b, p53, WBC count, sex, age, and CD10 status, several statistical tests were applied. Relations between the continuous variables were assessed by Spearman’s test for rank correlations. In addition, the continuous variables were categorised by splitting each into two groups (high and low) around the median, and then compared using Fisher’s exact test. For survival analysis, Kaplan-Meier step graphs were assembled and survival differences compared using the log rank test. The data were analysed with respect to p33ING1b nuclear and cytoplasmic expression, sex, age, WBC count, ALL phenotype, and CD10 status. All analysis was carried out using SPSS statistical software.

Immunohistochemistry

Multiple 5µm sections were cut from paraffin wax embedded, formalin-fixed normal and tumour tissue blocks. All sections were mounted on slides pretreated with APES in the conventional manner. A standard streptavidin–biotin–peroxidase complex (Dako, Ely, Cambridgeshire, UK) method was used. First, sections were dewaxed in xylene and then rehydrated by immersion in 99%, 95%, and 70% ethanol. Endogenous peroxidases were blocked by pretreatment of sections with a 0.5% solution of hydrogen peroxide in methanol for 10 minutes; the sections were then washed in running tap water. Antigen retrieval was achieved by pressure cooking for one minute in citrate buffer (200mM citric acid, 500mM NaOH, pH 6.6). Sections were then transferred into Tris buffered saline (TBS; 140mM NaCl, 50mM Tris/HCl, pH 7.6) for five minutes. The sections were then blocked with 10% normal rabbit serum in TBS for 10 minutes. Excess serum was removed and sections were covered and incubated at room temperature with the appropriate primary monoclonal antibody at the appropriate dilution for 60 minutes (GN1 at a 1/200 dilution and NCL-DO7 at a 1/20 dilution). Sections were washed twice with TBS for five minutes each, then incubated with the secondary biotinylated rabbit antimonouse antibody (Dako) at a 1/500 dilution for 45 minutes at room temperature. After this, the sections were washed twice for five minutes each with TBS, then covered with the tertiary streptavidin–biotin–peroxidase antibody (Dako) at a 1/100 dilution for 45 minutes at room temperature. After rinsing twice for five minutes each with TBS, the peroxidase activity was developed with 3,3′-diaminobenzidine (Sigma, Poole, Dorset, UK) 1 mg/ml in sterile distilled water-H2O2 solution for five minutes. The sections were then washed in tap water, counterstained with haematoxylin, then dehydrated and mounted in DPX.

Scoring

Tissue scoring was carried out by two observers, one an experienced histopathologist. Tissues were visualised by confocal light microscopy. The cell type was identified, and the proportions and intensities of nuclear and cytoplasmic staining for p33ING1b were estimated. In the case of p53, only nuclear staining was assessed. Intensity was assessed on a 4 point scale, as follows: 0, negative; 1, weak; 2, intermediate; and 3, strong staining. The percentage of cells staining was assessed on a 6 point scale, as follows: 1, 0–4%; 2, 5–19%; 3, 20–39%; 4, 40–59%; 5, 60–79%; and 6, 80–100%. The scores for intensity and proportion were then multiplied to give a composite score from 0–18, according to the method of Detre et al.17

RESULTS

General

The series comprised 89 boys and 56 girls between the ages of 0 and 14 years. One hundred and thirty four patients were classed as CD10 positive at diagnosis, whereas 31 were negative. The presenting WBC count for the whole series ranged from 1.4 to 1000 × 10⁹/litre, with a mean value of 48.94 × 10⁹/litre. There were 115 patients with B cell ALL (B-ALL), 16 with T cell ALL (T-ALL), nine with pre-B-ALL, three with non-B non-T-ALL, and two patients were classed as biphenotypic.

p33ING1b expression

Analysis of normal tissue showed strong uniform expression of p33ING1b in the nuclei of normal cells in bone marrow trephines (fig 1A). Strong nuclear expression was also seen in terminal deoxynucleotransferase positive precursor lymphoblastic cells in normal thymus and bone marrow (fig 1B). There was no cytoplasmic p33ING1b expression in the normal tissue. In contrast, p33ING1b was lost from the nuclei in 111 of the 145 patients with ALL (figs 1C,2), and replaced in all 111 cases by increased cytoplasmic expression of p33ING1b.

p53 expression

A sample of 68 cases was selected randomly, 34 of which were positive for p33ING1b, whereas the remaining 34 cases were negative. They were then assessed for the expression of p53 (fig 3). Most cases showed absent or weak labelling; only three cases in the series showed strong labelling (fig 1D).

Relations between variables

The data were analysed both as continuous and categorical variables. A positive correlation was observed between p33ING1b nuclear expression and p53 expression (r = 0.349; p = 0.0035), and inverse correlations were found between p33ING1b nuclear and cytoplasmic labelling (r = −0.445; p < 0.0001), and between p33ING1b cytoplasmic labelling and p53 labelling (r = −0.313; p = 0.0092). No other associations were found. Moreover, there was no difference in the proportion of cases showing loss of p33ING1b in T-ALL compared group.bmj.com on June 23, 2017 - Published by http://jcp.bmj.com/Downloaded from
with B-ALL, although it was found that all cases classed as pre-B-ALL or biphenotypic were p33\(^{ING1b}\) negative (table 1).

**Survival analysis**

Considering the series as a whole, low WBC count (\(p = 0.007\)), CD10 positivity (\(p = 0.029\)), age 4–6 at presentation (\(p = 0.07\)), and female sex (\(p = 0.09\)) were associated with a better survival. There was a trend towards improved survival in patients in whom ALL was assessed as nuclear p33\(^{ING1b}\) negative (\(p = 0.32\); fig 4).

**DISCUSSION**

Suppression of p33\(^{ING1b}\) has been associated with the loss of cellular growth suppression control and resistance to apoptosis, whereas its overexpression has been linked with the inhibition of cell proliferation and enhanced apoptosis.\(^{10}\) The involvement of p33\(^{ING1b}\) in the p53 signalling pathway provides a potential mechanism by which it may further function as a tumour suppressor. Loss of p33\(^{ING1b}\) has been shown to contribute to altered cell growth control and resistance to apoptosis.\(^{10}\) Nuclear localisation of p33\(^{ING1b}\) was first shown by Garkavtsev et al.\(^{2}\) and in our laboratory we have subsequently shown that p33\(^{ING1b}\) is expressed in the nuclei of most normal human cells and tissues.\(^{9}\)

In our study, p33\(^{ING1b}\) was lost from tumour cell nuclei in 78% of bone marrow trephines taken from patients with ALL at presentation. There was no significant difference in the proportion of cases of different phenotypes that showed loss of p33\(^{ING1b}\). Loss of nuclear p33\(^{ING1b}\) expression was associated with a higher cytoplasmic expression. Our results suggest a cellular compartment shift from the nucleus to the cytoplasm in ALL. p33\(^{ING1b}\) contains several important structural motifs, such as nuclear and nucleolar localisation signals and a PHD domain, which indicate that it functions in the nucleus.\(^{14-22}\) In addition, its ability to bind and modulate the transcriptional activity of p53 depends on nuclear localisation. Therefore, it seems likely that translocation of p33\(^{ING1b}\) from the nucleus to the cytoplasm would result in the loss of its tumour suppressor activity. The optimal function of p53 is dependent upon p33\(^{ING1b}\) and therefore loss of nuclear p33\(^{ING1b}\) would be predicted to compromise p53 function. This may be a factor in the deregulation of growth control that occurs in acute lymphoblastic leukaemia cells. It is possible that impaired function of p33\(^{ING1b}\) represents an alternative mechanism for abrogation of functional p53 in ALL. This hypothesis is supported by our finding of a weak but significant correlation between p33\(^{ING1b}\) and p53 expression, because p53 overexpression is associated with TP53 gene mutations. In our study, p53 overexpression was demonstrated in three of 68 cases. This is in keeping with the incidence of TP53 gene mutations in ALL at diagnosis.\(^{31}\) However, this would clearly require further analysis including TP53 sequencing because the relation between p53 immunostaining and mutations is not absolute.

“Our results suggest a cellular compartment shift from the nucleus to the cytoplasm in acute lymphoblastic leukaemia”
We are presently investigating a range of other types of tumours and an equivalent pattern of p33ING1b cellular compartment shift is evident in melanoma, thyroid, colon, and breast carcinomas. Others have reported changes in the subcellular localisation of p33ING1b, with a shift from the nucleus to nucleolus seen in HS68 fibroblast cell lines after exposure to UV light. Thus, it appears that reduced nuclear expression of p33ING1b may be important in several other human neoplasms. We have also cloned cDNAs corresponding to each of the reported ING1 mRNA isoforms into eukaryotic expression vectors and are currently investigating their independent functions.

The analysis of a variety of known prognostic factors in this series indicated that age 4–6 years, female sex, a low WBC count at presentation, and CD10 positivity were associated with a good outcome. Survival curves illustrating these data have been published previously. These results are consistent with other published studies. We found that 79.64% of patients with tumours that had lost nuclear p33ING1b survived compared with 78% of patients with tumours that had not lost nuclear p33ING1b. However, this was not significantly different in a log rank test (p = 0.32).
normal cellular function of the protein. These results support
with cytoplasmic expression. The shift in the subcellular
loss of nuclear expression of p33

Newcastle upon Tyne, NE1 4LP, UK
M M Reid,
A G Hall,
J Lunec,
Victoria Road, Newcastle upon Tyne, NE1 4PH, UK
Department, Royal Victoria Infirmary, University of Newcastle, Queens

REFERENCES
G S Nouman, J J Anderson, K M Wood, B Angus, Pathology
Department, Royal Victoria Infirmary, University of Newcastle, Queens
Victoria Road, Newcastle upon Tyne, NE1 4PH, UK
J Lunec, Cancer Research Unit, University of Newcastle
A G Hall, Paediatric Oncology Department, University of Newcastle
M M Reid, Haematology Department, Royal Victoria Infirmary, Newcastle upon Tyne, NE1 4LP, UK

Authors’ affiliations
G S Nouman, J J Anderson, K M Wood, B Angus, Pathology
Department, Royal Victoria Infirmary, University of Newcastle, Queens
Victoria Road, Newcastle upon Tyne, NE1 4PH, UK
J Lunec, Cancer Research Unit, University of Newcastle
A G Hall, Paediatric Oncology Department, University of Newcastle
M M Reid, Haematology Department, Royal Victoria Infirmary, Newcastle upon Tyne, NE1 4LP, UK

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chromosome mapping of a novel candidate tumor suppressor gene

Take home messages
• Nuclear expression of p33ING1b was lost in 78% of cases of
childhood acute lymphoblastic leukaemia (ALL) and was
associated with increased cytoplasmic expression of the
protein
• Patients with tumours that had lost nuclear p33ING1b demon-
strated a trend towards a better prognosis on Kaplan Meier
survival analysis
• Thus, the loss of nuclear p33ING1b expression may be an
important molecular event in the pathogenesis of childhood
ALL

Our study has shown that 78% of ALL cases studied showed
loss of nuclear expression of p33ING1b, which was associated
with cytoplasmic expression. The shift in the subcellular
localisation (nuclear to cytoplasmic) may cause the loss of
normal cellular function of the protein. These results support
the view that p33ING1b may function as a tumour suppressor,
and suggest that the loss of nuclear p33ING1b may be an
important molecular event in the pathogenesis of childhood acute
lymphoblastic leukaemia.

Figure 4: Kaplan Meier survival analysis for patients with acute lymphoblastic leukaemia (ALL) (n = 145) according to p33ING1b nuclear expression. A trend towards better survival was seen in patients who had lost p33ING1b nuclear expression (p = 0.32).

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EPO is for eosinophils as HNL is for neutrophils

Protein markers unique to particular types of inflammatory cells may help to diagnose certain respiratory diseases in future. Researchers have found that eosinophil peroxidase (EPO) is unique to eosinophils and human neutrophil lipocalin (HNL) unique to neutrophils by immunocytochemical staining of inflammatory cells in induced sputum and bronchial lavage (BAL) samples. By comparison, eosinophil cationic protein (ECP) was specific for eosinophils and neutrophils and myeloperoxidase (MPO) for neutrophils and monocytes. EPO and HNL did not show up in other cell types—lymphocytes, monocytes, macrophages, squamous cells, and ciliated epithelial cells.

Sputum and BAL samples were obtained from four healthy controls and 10 patients with a range of inflammatory respiratory disease—acute respiratory infection, asthma, chronic obstructive pulmonary disease, and prolonged cough. Washed cell pellets from each sample were spun onto microscope slides. The slides were incubated with monoclonal primary antibody—a cocktail of six antibodies for HNL and two antibodies separately for EPO, MPO, and ECP—then with alkaline phosphatase followed by antibody to alkaline phosphatase and counterstained. Care was taken to ensure optimum conditions for staining. Cell viability was shown by vital staining of pelleted cells to be consistently above 90%. Negative control slides were included, and a reference slide with differential staining with May–Grünwald–Giemsa was prepared for each sample.

ECP and MPO have previously been used extensively as markers for determining pathogenetic processes in respiratory disease according to the profile of inflammatory cells, though their specificity is not absolute.

β Topoisomerase is for lymphocytes, HNL is for neutrophils, and MPO is for monocytes.
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doi: 10.1136/jcp.55.8.596

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