High expression of Mcl-1 in ALK positive and negative anaplastic large cell lymphoma

R Rust, G Harms, T Blokzijl, M Boot, A Diepstra, J Kluiver, L Visser, S-C Peh, M Lim, W A Kamps, S Poppema, A van den Berg

Aim: To gain more insight into the genes involved in the aetiology and pathogenesis of anaplastic large cell lymphoma (ALCL).

Methods: Serial analysis of gene expression (SAGE) was undertaken on the CD4+ALK+ (anaplastic lymphoma kinase positive) ALCL derived cell line Karpas299 and as comparison on CD4+ T cells. Quantitative reverse transcription polymerase chain reaction (RT-PCR) and immunohistochemistry were performed on five ALCL derived cell lines and 32 tissue samples to confirm the SAGE data.

Results: High expression of Mcl-1 was seen in the Karpas299 cell line, whereas the two other antiapoptotic Bcl-2 family members, Bcl-2 and Bcl-XL, were not detected in the SAGE library. Quantitative RT-PCR confirmed the high expression of Mcl-1 mRNA and low expression of Bcl-2 and Bcl-XL in Karpas299 and in four other ALCL cell lines. To expand on these initial observations, primary tissue samples were analysed for Mcl-1, Bcl-XL, and Bcl-2 by immunohistochemistry. All 23 ALK+ and nine ALK− ALCL cases were positive for Mcl-1. Bcl-2 and Bcl-XL were expressed infrequently in ALK+ ALCL cases, but were present in a higher proportion of ALK− ALCL cases.

Conclusion: The consistent high expression of Mcl-1 in ALK+ and ALK− ALCL suggests that Mcl-1 is the main antiapoptotic protein in this disease. The high frequency of Mcl-1, Bcl-2, and Bcl-XL positive ALCL cases in the ALK− group compared with the ALK+ group indicates that ALK induced STAT3 activation is not the main regulatory pathway in ALCL.

Abbreviations: ALCL, anaplastic large cell lymphoma; ALK, anaplastic lymphoma kinase; β2m, β2 microglobulin; Ct, threshold cycle; RT-PCR, reverse transcription polymerase chain reaction; SAGE, serial analysis of gene expression.
Mcl-1 expression in ALCL

Figure 1  Real time reverse transcription polymerase chain reaction results for Bcl-2, Bcl-X<sub>L</sub>, and Mcl-1 in tonsil, CD4<sup>+</sup> T cells, and the Karpas299, SUP-M2, SR786, SU-DHL-1, and DEL cell lines. The bars on a logarithmic scale indicate the relative amount of mRNA. It is clear that Mcl-1 is the main antiapoptotic protein expressed in anaplastic large cell lymphoma derived cell lines.

SAGE
A detailed protocol for the SAGE procedure and a computer program (SAGE2000 version 4.12) for the analysis of gene specific tags were kindly provided by Dr KW Kinzler (John Hopkins Oncology Center, Baltimore, Maryland, USA). The SAGE procedure was performed as described previously. The SAGE libraries were compared and linked to the Unigene library to identify the corresponding genes.

Gene specific real time RT-PCR
Total RNA was isolated with Trizol (Life Technologies Inc, Gaithersburg, Maryland, USA) and first strand cDNA synthesis was performed using the protocol provided by the manufacturer (Life Technologies Inc). Assays-on-Demand<sup>®</sup> gene expression products (Applied Biosystems, Foster City, California, USA) were used for Bcl-2 (Hs00236808_s1), Bcl-X<sub>L</sub> (Hs00236329_m1), and Mcl-1 (Hs00172031_m1). Real time PCR was performed in 1× Taqman<sup>®</sup> Universal PCR master mix (Applied Biosystems); β2 Microglobulin (β2m) was used as a positive control and for normalisation; β2m forward (5'-gaaatggaagtccagtctctcgt-3'), β2m reverse (5'-ctgattggtgtctgcttgat-3'), and probe (5'-agtcatcctgcaacgcc-3') were dual labelled with 6-carboxyfluorescein (FAM) and 6-carboxytetramethylrhodamine (TAMRA). PCR was performed in triplicate in 1× qPCR master mix (Eurogentec, Liege, Belgium), using 900nM primers and 200nM probe. Reactions were performed on an ABI7900HT Sequence Detection System device (PE Applied Biosystems) using the standard program. Fluorescence was measured by means of sequence detection system software (SDS; version 2.0; Applied Biosystems). Mean cycle threshold values (Ct) and SDs were calculated for all genes. The amounts of the Bcl-2, Bcl-X<sub>L</sub>, and Mcl-1 targets were normalised relative to the amount of β2m target ($Δ$Ct = $Δ$Ct<sub>gene</sub> − $Δ$Ct<sub>β2m</sub>) and the SD of $Δ$Ct (SD($Δ$Ct)) was calculated (SD($Δ$Ct) = $\sqrt{(SD_{gene})^2 + (SD_{β2m})^2}$). The relative amount of target gene was measured by determining $ΔΔ$Ct ($ΔΔ$Ct = $Δ$Ct<sub>calibrator</sub> − $Δ$Ct<sub>sample</sub>) and the factor difference was calculated ($2^{−ΔΔCt}$). The range is given as $2^{−ΔΔCt + SD_{ΔΔCt}}$ and $2^{−ΔΔCt − SD_{ΔΔCt}}$.

Immunostaining
ALK, Bcl-2, Bcl-X<sub>L</sub>, and Mcl-1 positivity was assessed by immunohistochemistry using standard protocols and appropriate dilutions of monoclonal mouse anti-human ALK antibody, monoclonal mouse anti-human Bcl-2 antibody, polyclonal rabbit anti-human Mcl-1 antibody (Dako, Copenhagen, Denmark), and monoclonal rabbit anti-human Bcl-X<sub>L</sub> antibody (Zymed, San Francisco, California, USA). Peroxidase activity was visualised with diaminobenzidine and H<sub>2</sub>O<sub>2</sub>. For all analyses, negative controls (first incubation step without primary antibody) and positive control tissue sections were included. Differences in the number of positive cases between the ALK<sup>+</sup> and ALK<sup>−</sup> group were tested using Fisher's exact test.

RESULTS
We constructed gene expression profiles of Karpas299 and CD4<sup>+</sup> T cells using the SAGE technique. For Karpas299 we sequenced 10 678 tags representing 5090 different genes and for the CD4<sup>+</sup> T cells we obtained 8425 tags representing 4467 different genes. These expression profiles were compared and...
linked to the Unigene library to identify the corresponding genes. The SAGE tag belonging to the Mcl-1 gene was detected at a frequency of 0.04% in Karpas299, and 0.01% in the CD4+ T cells. The tag for the shorter splice variant of Mcl-1, was not detected in the SAGE libraries. No SAGE tags were seen for the other antiapoptotic members of the Bcl-2 family (Bcl-2 and Bcl-X\textsubscript{L}) in Karpas299 or in CD4+ T cells. To confirm this differential expression, ALK+ ALCCL cell lines Karpas299, SUP-M2, SR786, SU-DHL-1, and DEL were analysed using \textbeta\textsubscript{2m}, expressed at similar levels based on the SAGE libraries (0.29% in Karpas299 and 0.24% in CD4+ T cells), as a housekeeping gene. All five cell lines highly expressed Mcl-1 compared with tonsil and CD4+ T cells. Bcl-X\textsubscript{L} expression was moderate in the SUP-M2, SR786, SU-DHL-1, and DEL cell lines but was low in Karpas299 compared with CD4+ T cells and tonsil. Bcl-2 was slightly upregulated in the SUP-M2 and SR786 cell lines, but was reduced in the other ALCCL cell lines compared with the CD4+ T cells (fig 1).

Immunocytochemical staining for Mcl-1 revealed strong positivity in the Karpas299 cell line (fig 2A) and positive cytoplasmic staining in most of the tumour cells in all ALK+ and ALK– ALCCL cases. Staining for Bcl-2 was negative in all ALK+ cases, whereas four of the nine ALK– cases were positive. Bcl-X\textsubscript{L} was positive in eight of nine ALK+ cases, but only in three of 18 ALK+ cases (table 1; fig 2). The ALK– group included significantly more Bcl-2 (p = 0.004) and Bcl-X\textsubscript{L} (p = 0.000) positive cases than the ALK+ group.

**DISCUSSION**

Using SAGE analysis, the antiapoptotic member of the Bcl-2 family Mcl-1 was shown to be highly expressed in the ALCCL derived cell line Karpas299 compared with CD4+ T cells. In contrast, tags corresponding to the other two members of the antiapoptotic Bcl-2 family, Bcl-X\textsubscript{L} and Bcl-2, were not identified in the SAGE libraries. Screening of the SAGE library for tags corresponding to proapoptotic genes revealed the presence only of a low frequency of tags corresponding to the Bak gene. The SAGE results were confirmed with quantitative RT-PCR, which indicated increased expression of the Mcl-1 gene in Karpas299 and in the four other ALCCL derived cell lines. Moreover, quantitative RT-PCR for Bcl-X\textsubscript{L} and Bcl-2 clearly showed that expression was much lower than that of Mcl-1, consistent with the SAGE results.

Immunohistochemical analysis of 32 ALCCL cases (23 ALK+ and nine ALK–) demonstrated the presence of the Mcl-1 protein in most tumour cells in all cases. No difference in Mcl-1 staining intensity or percentage of positive tumour cells was seen between ALK+ and ALK– cases. Mcl-1 protein expression was reported previously in 10 of 10 and 10 of 11 ALCCLs. In a more recent study, Rasidakis et al detected Mcl-1 positivity in 16 of 26 ALK– ALCCLs using a 10% cutoff. These data confirm the consistent high expression of Mcl-1 and strongly suggest that Mcl-1, rather than Bcl-2 or Bcl-X\textsubscript{L}, is the main antiapoptotic protein of the Bcl-2 family expressed in ALCCL, and in particular in ALK+ ALCCL. Analysis of the apoptotic rate in ALCCL revealed low levels of apoptotic cells, ranging from 1.2% to 3.2%, in ALK+ and ALK– cases.

A possible role for Mcl-1 in lymphomagenesis is supported by the finding of a variety of lymphomas in Mcl-1 transgenic mice. Moreover, high Mcl-1 expression has been reported in various other lymphoma subtypes, including angioimmunoblastic T cell lymphoma, myeloma cell lines, cutaneous T cell lymphoma, diffuse large B cell lymphoma, and mantle cell lymphoma. Treatment with the cyclin dependent kinase inhibitor flavopiridol, which results in a strong downregulation of Mcl-1 expression, has been shown to be effective in multiple myeloma, leukaemia cell lines, and in chronic lymphocytic leukaemia samples, indicating the importance of Mcl-1 as an antiapoptotic protein.

"Our findings argue against a role for ALK induced activation of STAT3 and suggest involvement of other pathways leading to the induction of Bcl-X\textsubscript{L} and Bcl-2 expression"

The inhibition of JAK3 in two ALK+ ALCCL derived cell lines resulted in downregulation of activated STAT3, decreased levels of Bcl-2 and Bcl-X\textsubscript{L}, and no changes in Mcl-1 values. This suggests that other mechanisms are involved in the induction of Mcl-1 in ALCCL cases. However, in a more recent study Amin et al demonstrated the downregulation of Bcl-2, Bcl-X\textsubscript{L}, and Mcl-1 upon transfection of a dominant negative STAT3 in two ALCCL cell lines, supporting the STAT3 mediated induction of Mcl-1 in these cells. Similar results were obtained in macrophages and large granular lymphocyte leukaemia, which showed reduced Mcl-1 expression upon treatment with JAK inhibitors. Our data indicate the presence of the Mcl-1 protein in all ALCCL cases, independent of expression of the ALK protein, which suggests that besides the ALK induced activation of STAT3, other pathways might also contribute to the induction of Mcl-1 expression in ALCCL cases that lack ALK expression. This is supported by two studies that show the involvement of the phosphatidylinositol 3-kinase pathway in the upregulation of Mcl-1 expression.

In addition to positive regulation via survival signals such as activated STAT3 and the phosphatidylinositol 3-kinase pathway, Mcl-1 can also be downregulated via E2F1. Treatment with flavopiridol results in stabilisation of E2F1,
which acts as a transcriptional repressor of Mcl-1, and induces an effective downregulation of Mcl-1. Based on the balance between survival pathways and the effectiveness of Mcl-1 downregulation upon flavopiridol treatment, beneficial effects might be achieved by treatment of ALCL with flavopiridol.

As mentioned above, several studies suggest a role for ALK induced activation of STAT3 in the induction of Bcl-2 and Bcl-XL expression in ALCL cases. The consistent expression of Mcl-1 as demonstrated in our study indicates that treatment with agents such as flavopiridol may provide a novel tool for the treatment of both ALK+ and ALK− patients with ALCL.

ACKNOWLEDGEMENTS

This study was supported by the Foundation for Pediatric Oncology Research Groningen (SKOG, grant number 99-04).

Authors’ affiliations

R Rust, G Harms, T Blokzijl, M Boot, A Diepstra, J Kluiver, L Visser, S Poppema, A van den Berg, Department of Pathology and Laboratory Medicine, Universal Medical Centre Groningen and University of Groningen, 9700 RB Groningen, The Netherlands

S-C Peh, Department of Pathology, University of Malaya, 50603 Kuala Lumpur, Malaysia

M Lim, Department of Pathology, University of Utah Health Sciences Center, Salt Lake City, 84132 USA

W A Kamps, Department of Paediatric Oncology, Universal Medical Centre Groningen and University of Groningen

REFERENCES


Clinical Evidence – Call for contributors

Clinical Evidence is a regularly updated evidence-based journal available worldwide both as a paper version and on the internet. Clinical Evidence needs to recruit a number of new contributors. Contributors are healthcare professionals or epidemiologists with experience in evidence-based medicine and the ability to write in a concise and structured way.

Areas for which we are currently seeking authors:
- Child health: nocturnal enuresis
- Eye disorders: bacterial conjunctivitis
- Male health: prostate cancer (metastatic)
- Women’s health: pre-menstrual syndrome; pyelonephritis in non-pregnant women

However, we are always looking for others, so do not let this list discourage you.

Being a contributor involves:
- Selecting from a validated, screened search (performed by in-house Information Specialists) epidemiologically sound studies for inclusion.
- Documenting your decisions about which studies to include on an inclusion and exclusion form, which we keep on file.
- Writing the text to a highly structured template (about 1500–3000 words), using evidence from the final studies chosen, within 8–10 weeks of receiving the literature search.
- Working with Clinical Evidence editors to ensure that the final text meets epidemiological and style standards.
- Updating the text every six months using any new, sound evidence that becomes available. The Clinical Evidence in-house team will conduct the searches for contributors; your task is simply to filter out high quality studies and incorporate them in the existing text.
- To expand the topic to include a new question about once every 12–18 months.

If you would like to become a contributor for Clinical Evidence or require more information about what this involves please send your contact details and a copy of your CV, clearly stating the clinical area you are interested in, to Klara Brunnhuber (kbrunnhuber@bmjgroup.com).

Call for peer reviewers

Clinical Evidence also needs to recruit a number of new peer reviewers specifically with an interest in the clinical areas stated above, and also others related to general practice. Peer reviewers are healthcare professionals or epidemiologists with experience in evidence-based medicine. As a peer reviewer you would be asked for your views on the clinical relevance, validity, and accessibility of specific topics within the journal, and their usefulness to the intended audience (international generalists and healthcare professionals, possibly with limited statistical knowledge). Topics are usually 1500–3000 words in length and we would ask you to review between 2–5 topics per year. The peer review process takes place throughout the year, and our turnaround time for each review is ideally 10–14 days.

If you are interested in becoming a peer reviewer for Clinical Evidence, please complete the peer review questionnaire at www.clinicalevidence.com or contact Klara Brunnhuber (kbrunnhuber@bmjgroup.com).