CD2 Promoter Regulated Nucleophosmin-anaplastic Lymphoma Kinase in Transgenic Mice Causes B Lymphoid Malignancy

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Abstract. *Background: Nucleophosmin-anaplastic lymphoma kinase expression is associated with a lymphoid malignancy, anaplastic large cell lymphoma, and is characterized by a t(2;5) chromosomal translocation. Materials and Methods: We describe a novel transgenic mouse line in which NPM-ALK expression is targeted to the T-cell lineage using the CD2 promoter. Results: Surprisingly, the mice develop B cell lymphomas in the majority of cases. Conclusion: These data stress the importance of choice of promoter to drive transgene expression in obtaining the desired phenotype.*

Oncogenic tyrosine kinases are implicated in the pathogenesis of an array of malignancies and are in many cases generated as the result of chromosomal translocations (1). Nucleophosmin-anaplastic lymphoma kinase (NPM-ALK) is a kinase generated as the product of a t(2;5) translocation fusing the N-terminal region of nucleophosmin to the entire intracytoplasmic portion of ALK (2). NPM-ALK positive anaplastic large cell lymphomas (ALCL) are commonly of an activated T-cell phenotype expressing CD30, EMA and CD71, and often express perforin and granzyme B suggesting a cytotoxic T-cell origin (3, 4). In addition recent reports in the literature have described NPM-ALK$^+$ plasmablastic B-cell lymphomas in a minority of patients (5-7).

Attempts to generate a transgenic mouse model of ALK$^+$ ALCL have so far met with limited success in terms of replicating the phenotype of the human disease (8, 9). The choice of promoter used to drive NPM-ALK transgene expression is clearly critical in such models. The published transgenic models to date have used either the T-cell specific CD4 promoter or the pan-haematopoietic Vav promoter, generating mice which develop T-cell lymphoblastic-like lymphomas of a majority CD8$^+$ phenotype (with 50% of lymphomas also expressing CD30) in the former case and/or plasmablastic tumours following transgene expression using the Vav or CD4 promoters (8, 9). A recent publication describes the development of a transgenic mouse model expressing NPM-ALK under the regulation of the lck proximal promoter, in which 100% of the mice developed a large T-cell lymphoblastic lymphoma with a median latency of 8 weeks (10). However, the rapid development of tumours in these mice has prevented the breeding of a viable mouse line. In the present work we report the generation of a transgenic mouse line in which NPM-ALK is under the regulation of the T-cell specific CD2 promoter, which becomes active from the CD44$^-$CD25$^+$CD4$^-$CD8$^-$ stage in early thymic development (one T-cell development stage later than the lck proximal promoter and earlier than the CD4 promoter) (11). Given that specific windows of differentiation during cell development are thought to provide the correct epigenetic programming to permit tumorigenesis (12), we expected that the hCD2 promoter might drive transgene expression selectively in the T-lineage and induce T-cell transformation by switching on the hyperactive NPM-ALK tyrosine kinase at a critical stage during differentiation. In the event the CD2 promoter, like the Vav promoter, induced transformation in cells of the B- rather than T-lineage.

Materials and Methods

Cell line. The NPM-ALK positive human T-cell lymphoma line, Karpas-299 was maintained in RPMI-1640 plus glutamine supplemented with 10% FBS, penicillin and streptomycin.

Generation of NPM-ALK transgenic mice. The human NPM-ALK cDNA was isolated from the pcDNA$_3$NPM-ALK vector (from Prof. S. Morris, St. Jude Childrens Research Hospital, Memphis, TN, USA). To clone NPM-ALK into the CD2 promoter vector

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(11), blunt ends were created both 5' and 3' of the NPM-ALK cDNA in pcDNA3 followed by cloning into the SmaI site of the CD2 vector. Prokaryotic sequences were removed by digestion with ApaLI and ClaI to generate a 13.3 Kb fragment for microinjection. The 13.3 Kb fragment was injected into the pronuclei of eggs from C57BL/6J x CBA F1 mice and the micro-injected eggs were transferred to the ovarian ducts of pseudo-pregnant recipients.

Genotyping of mice. Mice were genotyped from tail tissue biopsies by both PCR and Southern blot. A 1.7 Kb BamHI fragment of $pcDNA_3$NPM-ALK was used as a probe for Southern blotting. PCR was carried out using primers specific for human NPM-ALK spanning the NPM-ALK fusion junction: NPM sense 5'-TCCCTTGGGGGCTTTGAAATAACACC-3', ALK antisense 5'-CGAGGTGCGGAGCTTGCTCAGC-3'.

Western blot analysis. Tissues were homogenised in 3% Brij-96 lysis buffer (20 mM $NaPO_4$, pH 7.5, 50 mM NaF, 0.1 mM Na_3VO_4, 150 mM NaCl, 10 mM EDTA, 10 mM EGTA and protease inhibitors) and proteins were separated by 8% SDS-PAGE. NPM-ALK presence was detected using a mouse monoclonal antibody to human ALK (ALK1) on polyvinylidene membranes (13).

Histopathological studies. When mice were found dead or moribund, autopsies were performed examining all tissues. Diseased tissue was fixed in 10% buffered formalin. Tumours were characterized immunophenotypically using the ABC-technique (DAKO, Hamburg, Germany). A wide panel of primary antibodies (purchased from Becton Dickinson Bioscience, Heidelberg, Germany and from DAKO, Hamburg, Germany or from Santa Cruz, Heidelberg, Germany) were used: B-cell markers included CD45R, CD20, pax5, CD38 and CD23, and T-cell markers included CD5, CD25, CD4, CD8 and CD3e. The polyclonal antibodies anti-lambda, anti-kappa, and anti-IgM, and secondary biotinylated goat anti-rabbit antibodies or rabbit anti-goat antibodies were obtained from DAKO. Stainings were visualized using diaminobenzidine (DAKO, Hamburg, Germany).

Results

The generation of NPM-ALK transgenic mouse lines. Seven lines of transgenic mice carrying the CD2/NPM-ALK transgene were generated using the construct illustrated in Figure 1A. Only one of these lines (line 4) developed tumours, the others were all healthy up to 24 months of age (Figure 1B). The development of T- and B-lymphoid cells was normal in the pre-tumorigenic cells of the transgenic mice (data not shown). NPM-ALK transgene expression was undetectable (by both RT-PCR and Western blotting) in all the lines except line 4, in which transgene expression was detectable only in tumours (Figure 1C).

A cohort of 82 mice from line 4 (43 transgene positive, 39 transgene negative) were housed in specified pathogen free conditions, until they became moribund up to a maximum of 24 months of age (Figure 1B, Table I). The first tumour was developed in a mouse of 9 months of age

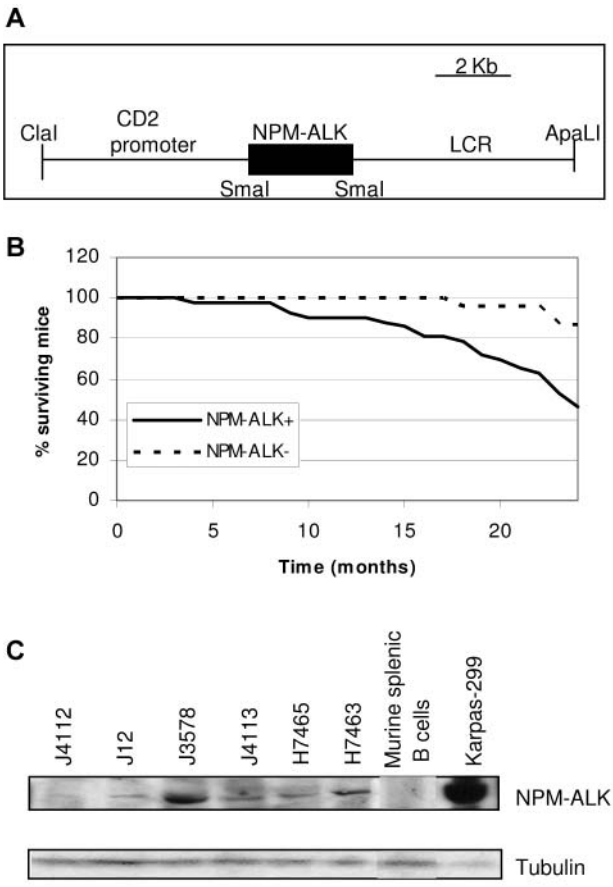

Figure 1. *Generation of NPM-ALK transgenic mice. (A). The human NPM-ALK cDNA was isolated from the $pcDNA_3$NPM-ALK vector (from Prof. S. Morris, St. Jude Childrens Research Hospital, Memphis, TN, USA) and was cloned into the CD2 promoter vector (LCR = locus control region) (11). (B). A survival curve demonstrating the long latency of disease progression in CD2/NPM-ALK transgenic mice. (C). NPM-ALK protein was only detectable in tumours, as shown here, and not in pre-tumorigenic thymocytes (data not shown). The numbers represent the animal identification tags.*

(Figure 1B). Mice that became ill (23 of the transgene positive mice) in this period, were examined for tumours and their phenotypes are summarised in Table I (some of the mice died at inconvenient times for analysis and hence their causes of death are unknown and are not reflected in Table 1). Of the transgene negative mice, 5 became moribund in the 2 year analysis period. These mice had no obvious tumour masses and the causes of death are unknown, although highly likely to be age-related. Transgene positive animals developed tumours in prominent abdominal lymph nodes, in the spleen, liver and/or thymus. Sometimes involvement of the peripancreatic/abdominal lymph nodes was detected. Liver tumours could also be seen by gross macroscopy.

Table 1: *Tumours develop in CD2/NPM-ALK transgenic mice. Specimens were fixed by immersion for 24 h in 10% buffered formalin before embedding in paraffin. Three to 5 mm-thick sections were cut and placed on glass slides. Tissue specimens were dehydrated, dewaxed and stained with haematoxylin-eosin (H+E) and Giemsa's solution.*

Mouse number	Age at diagnosis (months)	Pathology	Histological diagnosis
J4112	22	Distended abdomen containing liver tumour.	Chronic myelo-monocytic-acute myeloid leukaemia with sinusoidal and diffuse patterns.
J12	18	Thymic tumour, pale kidneys, slight splenomegaly.	T-cell rich diffuse large B cell lymphoma.
H9719	24	Liver tumour, splenic tumour, enlarged MLN.	Liver cell carcinoma + diffuse large B-cell (centroblastic) lymphoma in LN.
J1175	23	Enlarged abdomen containing liver tumour.	Extra-medullary haematopoiesis in the spleen. T-cell rich/histiocyte-rich diffuse large B cell lymphoma.
J3578	18	Enlarged liver and splenomegaly.	T-cell rich/histiocyte-rich diffuse large B cell lymphoma.
J4113	24	Enlarged MLN and splenomegaly	Nd
H7465	24	Spenic tumour.	Diffuse large B-cell lymphoma with a myelo-monocytic infiltrate in the spleen/liver.
H7463	24	Liver and MLN tumours.	Diffuse large B-centroblastic lymphoma.
J4108	18	Liver and lung tumours.	Follicular and diffuse large B- and T-cell/ histiocyte rich B-cell lymphoma.
H9725	19	Thymic tumour of 1 lobe, MLN tumour, PLN tumours.	Diffuse large B-cell lymphoma (centroblastic).
H7455	20	Splenic tumour, enlarged liver.	Nd
J4114	17	MLN and splenic tumours.	Nd
J13	19	Large liver tumour, splenomegaly.	Nd
J3575	14	Tumours of MLN, sub-mandibular LN, spleen, thymus slightly enlarged.	Nd
J492	24	Large splenic tumour, pale liver and kidneys.	Nd
J1566	24	Infiltration of tumours to kidney, MLN, PLN, spinal area, pale liver.	Nd
M1348	17	Large abdominal tumour unsure of origin, tumours along intestinal wall.	Nd

Nd = not determined, MLN = mesenteric lymph node, PLN = peripheral lymph node.

Histological analysis of tumour tissues. Histological analysis of the tumours revealed a range of distinct tumour phenotypes: myelo-monocytic leukemias, rare liver tumors (liver cell carcinomas) (data not shown) and malignant lymphomas (Figure 2a-d). The lymphoma phenotypes were predominantly of a diffuse large B-cell, but in some cases with follicular features (Figure 2a and b) and sometimes with features of centroblastic and T-cell-rich/histiocyte-rich B-cell lymphoma (Figure 2c and d). Tumour cells were also positive for ALK expression as shown by Western blotting (Figure 1C).

Discussion

We have developed a transgenic mouse line which develops lymphomas in the B-cell lineage expressing the NPM-ALK fusion tyrosine kinase. The lack of detectable expression of NPM-ALK pre-tumorigenesis suggests either that overall expression is very low and/or that expression is mosaic. The same situation pertains to the Vav-NPM-ALK transgenic lines that we have described previously (8). In contrast, NPM-ALK can be detected in the pre-tumorigenic tissue of transgenic mice expressing NPM-ALK under the regulation of the CD4 promoter (9). The reasons for these differences are not clear. However, it is possible that if expression of NPM-ALK was chimaeric at an early stage of thymic development, then this could lead to selective deletion of the transgene-positive cells, whereas at the later maturation stage at which the CD4 promoter becomes active, the thymocyte survival machinery might protect cells from deletion. This might also explain why the CD4 promoter-driven NPM-ALK is apparently more oncogenic than the same transgene expressed by either the Vav or CD2 promoters. It cannot be ruled-out that the effects we observe are due to the site of integration of the transgene and/or the aberrant/unchecked regulation of the NPM-ALK cassette, although the expression of NPM-ALK in the tumour tissues suggests otherwise. However, the lack of tumour development in the other lines produced points to the former, but due to the undetectable expression levels (possibly because NPM-ALK is not expressed in the other lines) a definitive conclusion cannot be drawn.

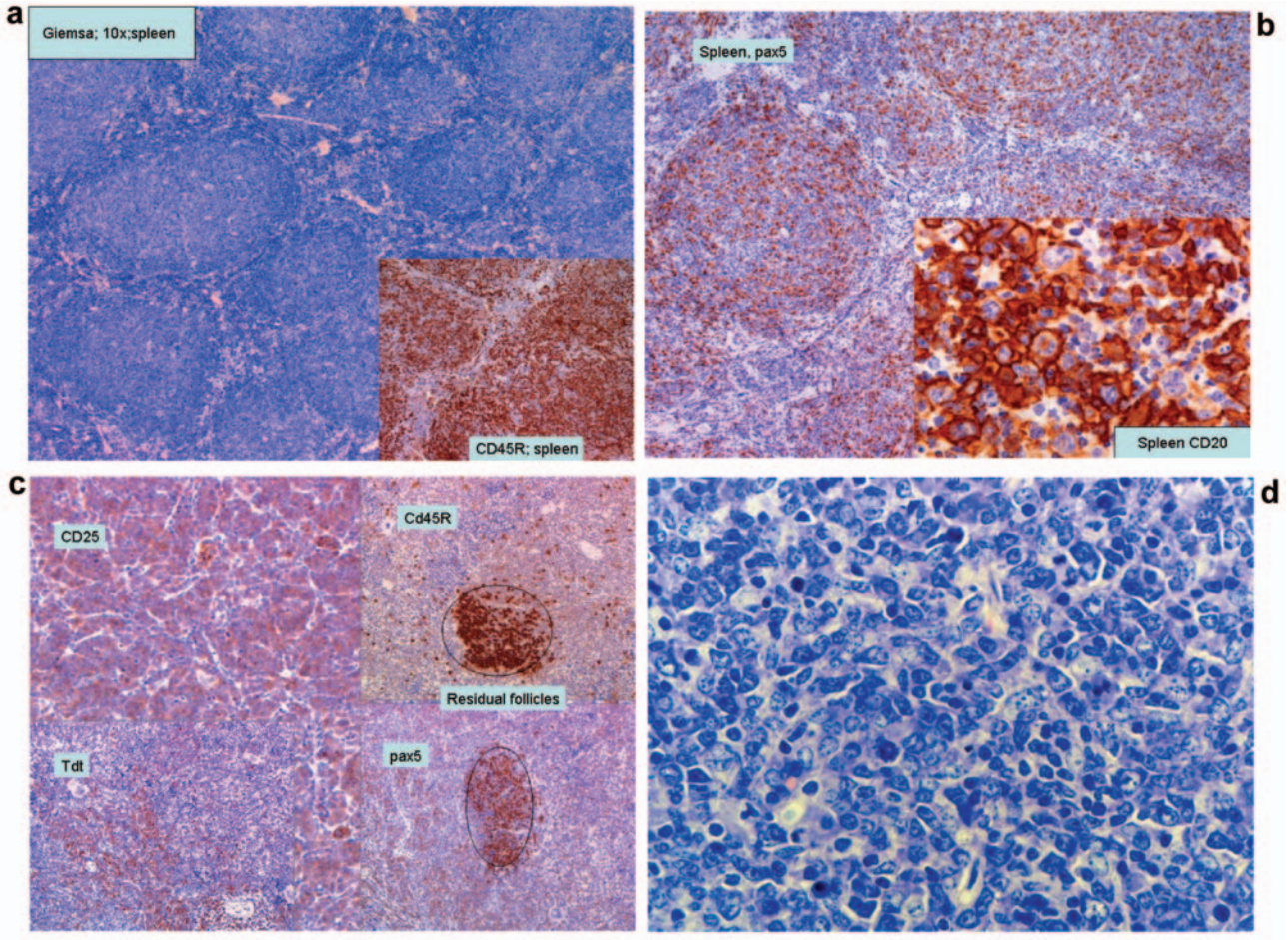

Figure 2. *CD2-NPM-ALK tumour histology. (a-d). Follicular and Diffuse Large B-Cell Lymphoma, sometimes with features of centroblastic and T-cell rich/histiocyte-rich B-cell lymphoma (a-c, stained as indicated, d, Giemsa).*

It was surprising that B-cell lymphomas developed in the majority of the transgenic mice, although the CD2 promoter has previously been shown to drive transgene expression in the B-lineage in some contexts (11). Other lineage-specific promoters to drive NPM-ALK expression in transgenic mice have likewise resulted in preferential transformation of the B-lineage, including the Vav promoter (14). Vav-promoter driven NPM-ALK gave rise to B-cell lymphomas of a plasmablastic phenotype, when the transgene copy number was high, compared to a putative peritoneal B1 B-cell phenotype in mice expressing a lower copy number, demonstrating preferential transformation of the B-lineage (8). In contrast the CD4 promoter, which drives expression from the $CD4^+CD8^+$ to $CD4^+$ stages of thymic development, gave rise to not only $CD8^+$ T-cell lymphomas, but also plasmacytomas, a phenomenon which is difficult to explain (9). Taken together with the present work, it appears that NPM-ALK needs to be expressed at a particular critical stage of T-cell development which has the correct epigenetic programming to enable the development of tumour cells characteristic of the human disease phenotype, in this case a phenotype similar to a cytotoxic T-cell. A striking example of the importance of stage-specific transgene expression is provided by the lck proximal promoter (active from an earlier $CD25^+CD44^-CD4^-CD8^-$ stage of thymic development), which was used to drive expression of the Rho GTPase inhibitor, C3 transferase in transgenic mice, causing cancer, a quite different non-transformed phenotype being observed when the CD2 promoter was used to drive expression very slightly later in development (15). It is, therefore, of particular interest that when the lck proximal promoter was used to drive NPM-ALK expression in transgenic mice, the mice developed a T-cell lymphoblastic lymphoma (10). The results from these mouse studies suggest that T-cells in particular require a developmentally regulated complex set of signals to enable transformation at particular stages. However, none of the models described to date faithfully mimics the human

disease phenotype suggestive of transformation of a more mature, or even an activated, T-cell.

In summary, we have developed a new transgenic mouse model of NPM-ALK lymphoma. Whilst this model does not replicate the human disease, it does provide a setting in which to explore the biochemical actions of the NPM-ALK oncoprotein and also to examine the efficacy of potential therapies. Our work demonstrates the importance of the choice of promoter when generating transgenic mouse models and the effects different promoters can have in the transgenic context.

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