Determining the contribution of *NPM1* heterozygosity to NPM-ALK-induced lymphomagenesis

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Heterozygous expression of Nucleophosmin (NPM1) predisposes to hematological malignancies in the mouse and cooperates with Myc in lymphomagenesis. NPM1 is therefore regarded as a haploinsufficient tumor suppressor. Heterozygous loss of *NPM1* occurs as a result of the t(2;5), which generates the oncogenic fusion tyrosine kinase, NPM-anaplastic lymphoma kinase (ALK), a molecule underlying the pathogenesis of anaplastic large cell lymphoma (ALCL). Given the aforementioned role of NPM1 as a tumor suppressor, we hypothesized that *NPM1* heterozygosity would cooperate with NPM-ALK in lymphomagenesis. In the event, we observed no difference in tumor latency, incidence or phenotype in NPM-ALK-transgenic mice heterozygous for *NPM1* relative to transgenic mice expressing both *NPM1* alleles. We propose that although the t(2;5) simultaneously reduces *NPM1* allelic dosage and creates the NPM-ALK fusion protein, the two events do not cooperate in the pathogenesis of ALCL in our mouse model. These data indicate that a tumor-suppressive role for NPM1 may depend on cellular and/or genetic context.

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Nucleophosmin (NPM)-1 is a ubiquitously expressed, multifunctional phosphoprotein attributed with both oncogenic and tumor-suppressive functions. The protein has a key role in several cellular processes related to the control of cell growth and proliferation, and is haploinsufficient in the maintenance of genomic stability, as well as being a haploinsufficient suppressor of hematological malignancies in the mouse.^{1,2} The t(2;5), generating the fusion tyrosine kinase NPM-anaplastic lymphoma kinase (ALK), is considered the driving event in greater than 80% of ALK⁺ anaplastic large cell lymphoma (ALCL) cases.³ However, although the role of NPM-ALK in cellular proliferation and tumorigenesis has been well documented, these studies have not considered a potential role for *NPM1* heterozygosity, occurring as a consequence of the t(2;5).

NPM1 heterozygosity accelerates disease onset in lymphoma-predisposed *Eμ-Myc* mice.² We therefore reasoned that *NPM1* heterozygosity may similarly cooperate with NPM-ALK in a transgenic mouse model of NPM-ALK-induced lymphomagenesis. However, no cooperation

between these two events was observed, thus providing evidence to dismiss a causal role for *NPM1* heterozygosity in the genesis of NPM-ALK⁺ ALCL in this model system.

MATERIALS AND METHODS

Mice

The generation of *CD2/NPM-ALK* transgenic mice was described previously as was the generation of *NPM1* heterozygous mice.^{2,4} All mice were maintained on a C57BL/6J background (Jackson Laboratory, Charles River, Margate, UK). Mice were housed under specific pathogen free conditions in a barrier facility at the University of Cambridge, UK.

Histology and Immunohistochemistry

Mice displaying clinical signs were euthanized and post-mortem performed examining all tissues. Tissue sections were stained with hematoxylin and eosin for histological examination. Immunohistochemical analysis was performed with the following antibody: ALK (Invitrogen, Paisley, UK).

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Western Blot

Analysis of protein expression by western blot was performed as described previously⁵ using antibodies specific to NPM1 (NA24) (Abcam, Cambridge, UK) and actin (Sigma). Densitometric analysis of protein expression levels as a factor of the respective actin-loading control was performed using AIDA software and a Fuji LAS 4000 chemilluminescence analyzer (Raytek, Sheffield, UK).

Statistics

Kaplan–Meier survival curves were produced using MedCalc software (Mariakerke, Belgium).

RESULTS

To examine the impact of *NPM1* genetic heterozygosity on the incidence, latency and pathological features of NPM-ALK-induced tumorigenesis, *CD2/NPM-ALK⁺* mice were crossed with *NPM1^{+/-}* mice and the offspring (34 *CD2/NPM-ALK⁺NPM1^{+/-}* and 25 *CD2/NPM-ALK⁺NPM1^{+/+}* mice), as well as 39 genetically wild-type mice (littermates obtained from maintenance of the *CD2/NPM-ALK* mouse colony) and 12 *NPM1* heterozygous mice, were monitored over a 2-year period (Figure 1 and Table 1). Of the 25 *CD2/NPM-ALK⁺NPM1^{+/+}* and 34 *CD2/NPM-ALK⁺NPM1^{+/-}* mice under observation, 11 (44%) and 13 (38%) were

euthanized within the 2-year period, respectively (Figure 1a and Table 1). Lymphoma developed in both populations of NPM-ALK-expressing mice after a long latency that ranged from 10 to 24 months (Table 1). During this time, five (13%) transgene-negative wild-type mice displayed clinical signs and were culled. There was no evidence of tumors and the cause of ill-health remains unknown but is highly likely to be age-related. Although *CD2/NPM-ALK⁺NPM1^{+/+}* and *CD2/NPM-ALK⁺NPM1^{+/-}* mice succumbed to disease significantly earlier than wild-type mice (Figure 1a; *CD2/NPM-ALK⁺NPM1^{+/+}* versus wild-type mice, $P = 0.01$; *CD2/NPM-ALK⁺NPM1^{+/-}* versus wild-type mice, $P = 0.02$), the survival distribution of *CD2/NPM-ALK* transgenic mice expressing one or both alleles of *NPM* was equal (Figure 1a; *CD2/NPM-ALK⁺NPM1^{+/+}* versus *CD2/NPM-ALK⁺NPM1^{+/-}* mice, $P = 0.7$). Thus, heterozygous disruption of the *NPM1* locus does not significantly accelerate disease onset or increase incidence in the context of oncogenic NPM-ALK signaling.

Table 1 details the macroscopic and microscopic pathology for each mouse that was culled on presentation of clinical signs or died unexpectedly (≤ 2 years of age). At necropsy, many of the mice that died prematurely presented with enlarged lymph nodes with or without splenomegaly. In tissue sampled from mice of either genotype, histological analysis revealed the presence of extensive infiltrates characterized by atypical lymphocytes in spleen and lymph nodes that disrupted the normal tissue architecture (Figure 2a). Immunohistochemical analysis with an anti-ALK antibody confirmed expression of NPM-ALK in the tumor tissue (Figure 2b). Furthermore, western blot analysis revealed that loss of heterozygosity for *NPM1* was not a feature of these tumors as NPM1 expression could be detected in tumors arising from both *NPM1* heterozygous and *NPM1* wild-type mice (Figure 2c). Amplification of NPM1 expression was likewise not a feature of the tumors that arose in the *CD2/NPM-ALK⁺NPM1^{+/-}* mice, as protein lysates prepared from these tumors contained on average lower levels of NPM1 than tumor lysates extracted from *CD2/NPM-ALK⁺NPM1^{+/+}* mice (average densitometry level = 0.32 compared with 2.24; Figure 2c). B-cell lymphoma was the predominant tumor type presented by the *CD2/NPM-ALK⁺NPM1^{+/+}* mice, occurring in five (20%) mice, and was the only tumor type that developed in the *CD2/NPM-ALK⁺NPM1^{+/-}* mice, occurring in six (18%) of the mice (Table 1 and Figure 1b). Although low, the incidence of lymphoma was significantly different between the WT and the *CD2/NPM-ALK⁺NPM1^{+/-}* cohorts ($P < 0.01$) but not between the *CD2/NPM-ALK⁺NPM1^{+/-}* and the *CD2/NPM-ALK⁺NPM1^{+/+}* groups ($P = 0.8$; Figure 1b). A predominance to develop B-cell lymphomas is consistent with the tumor phenotype previously described for the *CD2/NPM-ALK⁺NPM1^{+/+}* transgenic mice, although an incidence of 20% is lower than published observations.⁴

Figure 1 Survival and lymphoma incidence of *CD2/NPM-ALK⁺NPM1^{+/+}* and *CD2/NPM-ALK⁺NPM1^{+/-}* mice. Kaplan–Meier analysis of survival (a) and lymphoma incidence (b). *P*-values indicate the significance of survival or lymphoma incidence between two populations (indicated by brackets) and were assessed using the log-rank test. $P < 0.05$ is considered to be significant.

Table 1 Disease latency, incidence and pathology for *CD2/NPM-ALK⁺NPM1^{+/+}*, *CD2/NPM-ALK⁺NPM1^{+/-}*, *NPM1^{+/-}* and *NPM1^{+/+}* mice

Genotype	ID	Age (months)	Macroscopic pathology	Microscopic pathology/histological diagnosis
<i>CD2/NPM-ALK⁺NPM1^{+/+}</i> <i>n</i> = 11/25 (44%)	271	10	NAD	Chronic inflammatory infiltrate in the kidney
	273	14	Splenomegaly	Massive splenic red pulp expansion
	798	18	Enlarged heart and inguinal LN	Patchy lymphoid infiltrate in lung (tumor); reactive lymph node with plasmacytosis
	780	19	Enlarged MLN, SMLN and thymus; splenomegaly	Lymphoma in LN, spleen and kidney
	268	19	Enlarged MLN, lung, spleen and liver	Diffuse large cell lymphoma
	781	20	Weight loss; pale liver	NAD
	786	20	Pale kidneys; distended stomach	Splenic angioma and glomerulonephritis
	800	20	Hepatomegaly	Hepatocellular carcinoma with attached epithelial tumor
	792	22	Enlarged ILN; splenomegaly; pale kidneys	Lymphoma in LN and spleen
	1178	23	Splenomegaly; hepatomegaly	Hepatocellular carcinoma; histiocytic carcinoma in spleen and liver
	1164	24	NAD	Lymphoma in LN (possible centroblastic B-cell lymphoma)
<i>CD2/NPM-ALK⁺NPM1^{+/-}</i> <i>n</i> = 13/34 (38%)	269	16	Enlarged MLN and thymus	Splenic lymphoma
	270	16	NAD	Focal inflammatory infiltrate in muscle with probable vasculitis
	788	17	Enlarged PLN	Diffuse large cell lymphoma in LN and spleen
	777	18	NAD	NAD
	779	19	Enlarged ILN and MLN	Lymphoma in LN
	783	19	NAD	NAD
	778	20	NAD	Iron accumulation in LN and spleen
	785	20	Splenomegaly; pale liver	Lymphoma in spleen and lung
	790	20	Enlarged ILN and MLN	Diffuse large cell lymphoma in LN and spleen; hemangioma
	797	21	Fluid in thoracic cavity; pale kidneys	Glomerulonephritis
	207	23	NAD	NAD
	185	24	NAD	Fatty change in liver
	787	24	Enlarged MLN	Large cell lymphoma in LN and spleen
<i>NPM1^{+/+}</i> (wild-type) <i>n</i> = 5/39 (13%)		18	NAD	NAD
		18	NAD	NAD
		23	NAD	NAD
		23	NAD	NAD
		23	NAD	NAD

Table 1 Continued

Genotype	ID	Age (months)	Macroscopic pathology	Microscopic pathology/histological diagnosis
NPM ^{+/-} <i>n</i> = 6/12 (50%)	37	16	Pale kidney; enlarged ILN	NAD
	33	17	Cachexia	NAD
	24	17	Sore on neck	NAD
	382	19	Cachexia; hepatomegaly	Low-grade hepatoma; glomerulonephritis
	11	8	Abcess in groin	Lymphadenitis with neutrophils
	58	10	NAD	NAD

NAD = no abnormality detected; LN = lymph node; MLN = mesenteric lymph node; ILN = inguinal lymph node; SMLN = sub-mandibular lymph node; BM = bone marrow.

n relates to the number of mice presenting with an abnormal pathology among the cohort of mice of the specified genotype.

Other tumor types detected in the CD2/NPM-ALK⁺NPM1^{+/+} mice were largely non-hematological and included two cases of hepatocellular carcinoma (Table 1). However, one mouse (no. 1178) in addition to developing hepatocellular carcinoma presented with histiocytic sarcoma in the liver and spleen. Among both CD2/NPM-ALK⁺NPM1^{+/+} and CD2/NPM-ALK⁺NPM1^{+/-} cohorts, a small number of mice presented with an abnormal pathology in non-lymphocytic cells that was not associated with tumor formation, and therefore presumably unrelated to NPM-ALK expression, and possibly age-related (Table 1 and Figure 1a). These abnormal pathologies included inflammatory infiltrate in the heart and glomeruli of the kidney, as well as extensive red pulp expansion of the spleen. Finally, one CD2/NPM-ALK⁺NPM1^{+/+} and three CD2/NPM-ALK⁺NPM1^{+/-} mice could not be associated with any distinct pathology following histological examination and therefore the reason for ill-health remains unknown (Table 1). Importantly, of those tumors for which NPM-ALK expression could be detected no significant difference in the incidence was observed between the two populations of mice (Figure 1b), indicating that NPM1 heterozygosity does not cooperate with NPM-ALK in lymphomagenesis or antagonize its oncogenic function in this model.

Although the CD2/NPM-ALK transgenic mice are prone to developing lymphoid malignancies,⁴ the NPM1^{+/-} genotype is reported to chiefly predispose to myeloid malignancies (either myeloproliferative disease-like myeloid leukemia (MPD-like ML) or ML).² MPD-like ML in these mice is characterized by leukocytosis together with a vast increase in the number of well-differentiated myeloid cells in the bone marrow, spleen and non-hematopoietic tissues.² ML is characterized by severe anemia, mild leukocytosis and thrombocytopenia, as well as the presence of leukemic blasts in affected tissues.² Of the CD2/NPM-ALK⁺NPM1^{+/+} and CD2/NPM-ALK⁺NPM1^{+/-} mice for which blood counts were taken, all mice exhibited

perturbations in at least one of the following blood parameters: white blood cell, red blood cell or platelet count or hemoglobin levels (compared with the range obtained from the peripheral blood of 10-month-old wild-type litter mate mice (*n* = 10); Supplementary Table S1 and Supplementary Figure S1). However, the blood counts were not consistent with a diagnosis of MPD-like ML or ML. Extreme expansion of myeloid cells was not a feature of any of the tissues examined, which included bone marrow and analysis of blood smears (data not shown), and hence, no evidence of myeloid malignancy was observed in any of the mice, regardless of NPM1 genetic dosage. Equally, there was no evidence of myeloid disease in the NPM1^{+/-} cohort (Supplementary Table S1). In none of the blood samples analyzed was there any trend to indicate a common deficiency or increase in blood cell count parameters in any of the cohorts, and group readings did not differ significantly from one another (Supplementary Figure S1). This disparity with the literature may reflect generation-specific differences, subtle changes in the genetic background of the mice following outcross to the NPM-ALK transgenic lines or disparities in housing of the mice compared with the original mouse colonies.

DISCUSSION

Although transgene expression of NPM-ALK is sufficient to drive lymphoma development in several mouse models of NPM-ALK-induced tumorigenesis, none of these models faithfully recapitulate the human disease.⁴⁻⁹ For instance, disease latency is in most cases long and manifests after many months, suggesting the need for additional cooperating events to accelerate disease. In addition, in these and other models of NPM-ALK-driven lymphomagenesis, NPM-ALK-expressing B-cell lymphomas frequently develop despite supposed T-cell-restricted expression of the transgene.^{4,6} In all of these models, a potential role for t(2;5)-associated NPM1 allelic loss in tumor development has not been

Figure 2 Histopathological presentation of tumors. (a) Hematoxylin and eosin-stained spleen and lymph node sections from *CD2/NPM-ALK⁺NPM1^{+/+}* and *CD2/NPM-ALK⁺NPM1^{+/-}* mice presenting with tumor masses showing atypical lymphoid infiltrate disrupting the normal tissue architecture. (b) Immunohistochemical analysis of tumor sections to detect NPM-ALK expression. (c) Western blot analysis of NPM and actin expression levels in tumors isolated from *CD2/NPM-ALK⁺NPM1^{+/+}* and *CD2/NPM-ALK⁺NPM1^{+/-}* mice compared with *NPM^{+/-}* and *NPM^{+/+}* MEFs. The numbers refer to densitometric analysis of NPM expression levels in comparison to the respective actin control.

considered. Here, we addressed this issue by crossing mice expressing NPM-ALK from the *CD2* promoter with *NPM1* heterozygous mice and examining the resultant offspring. This mouse model was chosen because of the long latency to disease development and a lower disease penetrance, and hence, to provide a clear window in which our hypothesized increased disease incidence and shorter latency owing to a *NPM1* heterozygous background could be observed.⁴ In the event, no difference in tumor latency, incidence or phenotype was observed, providing evidence that heterozygous expression of *NPM1* does not cooperate with NPM-ALK in tumorigenesis in this model system. These results differ to the published observations that oncogenic Myc expression cooperates with *NPM1* heterozygosity to accelerate tumor onset in lymphoma-predisposed mice.² We propose that whereas Myc and *NPM1* heterozygous expression have complementary functions in oncogenesis that permit cooperation, this is not the case for NPM-ALK expression and *NPM1* heterozygosity although this remains to be determined by complementary *in vitro* studies. These findings are in accordance with analyses of *ALK⁺* ALCL patient data, indicating that tumors harboring *ALK* fusions where the partner gene is not *NPM1* exhibit an immunophenotypic, clinical and prognostic behavior that is indistinguishable from that of classical t(2;5) ALCL tumors,¹⁰ although the t(2;5) is the most common translocation, observed in greater than 80% of cases raising the question as to why this particular event is selected for.¹¹ However, together, these data indicate that *NPM1* heterozygosity does not contribute to the genesis of t(2;5)-induced ALCL. It seems likely that the significance of this translocation lies largely, if not entirely, in the creation of an oncogenic tyrosine kinase that drives tumorigenesis. However, before this statement can be firmly concluded, it is necessary to examine the consequences of *NPM1* heterozygosity in an *NPM-ALK* transgenic model with a more penetrant disease phenotype or even a mouse in which NPM-ALK expression and *NPM1* heterozygosity are achieved simultaneously, for example, using the translocator system developed by Rabbitts *et al.*¹² Yet, one must consider that the phenotype observed in mouse models of cancer is influenced by a number of tangible and intangible variables and as such a direct correlation between mouse and human pathologies is often difficult to make, although we have observed that human NPM-

ALK forms some of the same hetero-complexes and occupies the same cellular sub-compartments in murine cells as has been observed in human cells (Supplementary Figure S2), suggesting that at least at the molecular level, NPM-ALK biology is equivalent in both mouse and human.

Supplementary Information accompanies the paper on the Laboratory Investigation website (<http://www.laboratoryinvestigation.org>)

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DISCLOSURE/CONFLICTS OF INTEREST

The authors, SDT, FKEM, CEH, BJH and RT have no conflicts of interest to disclose.

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