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Twenty years of modelling NPM-ALK-induced lymphomagenesis

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1. ABSTRACT

Our current understanding of oncogenic Anaplastic Lymphoma Kinase (ALK)-induced lymphomagenesis has relied for over 20 years on multiple and complementary studies performed on various experimental models, encompassing ALK oncogene expressing cells, their grafts into immune-compromised mice, the generation of genetically engineered mouse models (GEMMs) and, when available, the use of patient samples from Anaplastic Large Cell Lymphoma (ALCL) tumour banks. Of note, and to our knowledge, no ALK-positive ALCL 3D culture system has been described so far. In this review, we will first outline how these different cell and mouse models were designed, and what key findings they revealed (or confirmed) towards oncogenic ALK-induced lymphomagenesis. Secondly, we will discuss how recent and revolutionary advances in genetic engineering technology are likely to complete our understanding of ALK-related diseases in an effort to improve current therapeutic approaches.

2. INTRODUCTION

The discovery of the t(2;5)(p23;q35) encoding Nucleophosmin-Anaplastic Lymphoma Kinase (NPM-ALK) some 20 years ago signalled the start of considerable research efforts to determine the mechanism(s) by which this aberrantly expressed protein might induce lymphoma development (1). In the intervening period, oncogenic forms of ALK have been implicated in many further forms of cancer and activated by a variety of mechanisms, some still to be elucidated (2). Indeed, the common presence of activated ALK/ALK fusion proteins in many malignancies begs the question as to whether there are common underlying mechanisms and/or tissue-specific effects. In this regard the development of model systems has been instrumental to our research efforts as it has been to cancer research in general although, as with most model systems there remain many caveats particularly when considering the tumour as a mini-organ with its own blood supply, stroma and inflammatory milieu to name but a few assets. In this review we will discuss our current understanding on the biology of ALCL as gleaned from model systems generated to mimic some aspects of ALCL.

3. CONFIRMATION OF THE ONCOGENIC POTENTIAL OF NPM-ALK

3.1. *In vitro* studies

The first port of call for many studies into mechanisms of oncogenesis commence with cell line model systems, either those created from patient-derived tumours or exogenously expressed oncogenes in established cell lines. An extensive literature on *in vitro* ALK-oncogene signalling has been carried out using cell line models that were and still are useful for the biochemical analysis of the classical and newly proposed cancer hallmarks (2, 3). Initial studies sought to confirm the oncogenic potential of the newly identified NPM-ALK protein by expressing it in rat1a fibroblasts and IL3-dependent BaF3 B-cells. In both cases features of transformation were observed including anchorage-independent growth, foci formation and in the latter case independence from IL3-mediated signalling (4, 5). However, ALCL is a T-cell malignancy and therefore whilst it had been shown that NPM-ALK was able to induce some of the hallmarks of cancer *in vitro*, its relevance to T-cell biology was lacking. In this regard, effects of exogenous expression in the Jurkat T-cell leukaemia line was investigated but given that this cell line is already transformed, data were less informative and more restricted to understanding the signalling pathways that could be activated by NPM-ALK in a T-cell context (6). More recently, NPM-ALK expressing cell lines have been established from lentivirally transduced primary human mature $CD4^+$ T-cells (7). These have been reported to faithfully mimic key ALCL characteristics (increased cell volume and biomarker expression (CD30, Ki-67) as well as expression of immunosuppressive molecules (IL-10, PD-L1, STAT3/mTORC1 signalling activation)) (7). ALK oncogene mediated transformation of these cells relies, at least in part, on the use of the pre-existing IL-2 dependent signalling pathway (8). Importantly, this study demonstrates that the ALK oncogene is able to transform the cell type that is considered its cell of origin, i.e. normal T lymphocytes, thus providing the first cell system allowing *in vitro* studies both on ALK oncogene-mediated early mechanisms of lymphoma development and on the effects of early (new and/or combined) therapeutic interventions (9). However, all these cellular models more or less have limitations: as just described, they do not exactly recapitulate the T cell lineage characteristics of ALK-positive ALCL (except in the latter case) which also compromises their use for studying early stages of lymphomagenesis and disease progression mechanisms. In addition, all models remain *in vitro* experimental tools which obviously do not recapitulate the impacts of environmental factors known to influence tumour development and response to therapy (tumour and stromal cell interactions, immune system contribution, angiogenesis etc.). Thus, over the years and as in many others cancer studies (10), the mouse has emerged as the model organism of choice to study ALK-induced tumorigenesis including transgenic mouse models as well as murine chimeras and xenotransplantation models (human cell lines or patient material transplanted in most cases sub-cutaneously into immune deficient mice).

3.2. *In vivo* studies

The generation of murine chimeras whereby bone marrow is transduced to express the oncogene of interest before transplantation into irradiated recipient mice is often the first step towards providing evidence of *in vivo* oncogenic potential. Indeed, the oncogenic potential of NPM-ALK *in vivo* was first shown in such a system whereby murine bone marrow cultures in the presence of stem cell factor and IL6, were retrovirally transduced to express NPM-ALK before implantation into irradiated recipient mice (11). Perhaps not surprisingly (due to the B-cell skew in culture conditions), these mice developed B-lymphoid malignancies but nonetheless provided the first *in vivo* evidence of the oncogenic potential of NPM-ALK. Many other murine models have since been generated confirming the *in vivo* oncogenic potential of NPM-ALK and these are discussed further below.

4. DISSECTING NPM-ALK SIGNALLING PATHWAYS

The large majority of studies have used a combination of cell lines including patient-derived established cell lines to examine the oncogenic effects of NPM-ALK, in particular with a view to the signalling pathways in which it is involved and/or activated (12) (these have been reviewed extensively elsewhere and will not be discussed here (2, 13)). The most highly cited ALCL cell lines in use include Karpas-299, SUDHL-1, SUP-M2, SR786 and DEL all available from tissue banks and largely retrospectively identified as being ALK^+ ALCL (see Table 1) (14). However, these are generally isolated from patients with advanced and/or aggressive disease and together with serial propagation *in vitro* over many years, the inherent (epi)genetics are less likely to mimic those observed in the original tumour (15). For example, the Karpas-299 cell line has a deletion of *PTEN* as well as mutation of *TP53* and it is not clear whether these additional genetic changes were present in the original tumour or gained through *in vitro* serial propagation (16). Moreover, their culture within different media and serum conditions, according to empirical use in any given laboratory, are likely to induce many cellular drifts that could potentially change their behaviour and response to any given insult. Therefore, whilst these cell lines have provided the workhouses for biochemical studies into NPM-ALK activity, these data must be interpreted with caution and backed-up in additional model systems. In this regard, a number of murine models have been utilised to assess the biochemical pathways driving tumour growth in tumours arising *de novo*. In particular, the roles of signalling proteins such as STAT3 and JNK have been confirmed in murine models (17-19).

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Table 1. A non-exhaustive list of models used to investigate the biology of NPM-ALK induced lymphoma

Model system	Examples	Key findings	Refs
Cell lines engineered to express NPM-ALK	BaF3, Rat1a, MEFs, NIH3T3, 32D, Jurkat, 293TRex Tet ON, MEF Tet OFF	Growth factor independence, anchorage-independent growth, foci formation	(4-6, 37, 64-73)
Patient-derived cell lines	ALK$^+$: Karpas-299, SUDHL-1, DEL, SUP-M2, SR-786, COST, PIO, L82, KI-KJ, JB6, TS (variant of SUDHL-1 carrying a tet-inducible shRNA against ALK), ALK$^-$: FEPD, Mac-1, Mac-2a, Mac-2B, JK	Many NPM-ALK signalling pathways, NPM-ALK signalosome	(14, 69, 74-88)
Primary T-cells transformed by NPM-ALK	Human peripheral blood derived CD4$^+$ T-cells	First evidence of NPM-ALK induced transformation of mature human T-cells	(7, 8)
GEMMs	CD4/NPM-ALK, VAV/NPM-ALK, CD2/NPM-ALK, LCK/NPM-ALK, EμSRα-tTA/Tet-O-NPM-ALK	Proof of the oncogenic potential of NPM-ALK in the lymphoid compartment	(17, 18, 23, 24, 29)
Chimeras	Retroviral transduction of murine bone marrow from wild-type, Cre or other transgenic lines with NPM-ALK followed by implantation into irradiated recipients	Proof of the oncogenic potential of NPM-ALK in the lymphoid compartment	(11, 20, 21, 28)
Xenografts	ALCL cell lines implanted sub-cutaneously into immune deficient mice, patient derived primary tumours implanted sub-cutaneously or intra-peritoneally into immune deficient mice	Therapy and tumour heterogeneity studies	(33, 35, 36, 38-40, 43, 54, 89, 90)
NPM-ALK: Nucleophosmin-Anaplastic Lymphoma Kinase			

5. ELUCIDATING MECHANISMS OF TRANSFORMATION AND THE CELL OF ORIGIN

5.1. Role of murine chimeras

Since the initial study of Kuefer *et al* described above (11), refinements of the chimeric system have been made in an attempt to produce a murine mimic of ALCL. In particular, tweaking of the system has been partially successful in recapitulating ALCL *in vivo*; transduction of IL9 transgenic mouse (which are predisposed to thymic T-cell lymphomas) bone marrow before implantation into recipient mice resulted in mice developing some T-lymphoblastic lymphomas (T-LBL) but also plasmacytomas and plasmablastic diffuse large B-cell lymphomas (20); the multiplicity of infection (MOI) has also been shown to impact on disease phenotype with high levels (>5) resulting in the rapid development of polyclonal histiocytic disease and low MOI (<0.005) in later onset monoclonal B-lymphoid malignancies (21); use of bone marrow from cre reporter transgenic mice such as Lysozyme M-cre and Granzyme B-cre transduced with a Lox-STOP-Lox-NPM-ALK encoding vector implanted into recipient mice led to histiocytic malignancies and T-LBL/histiocytic disease respectively (22). This latter system has the potential to result in a murine mimic of ALCL dependent on promoter driven-Cre usage and it remains to be seen the effects of driving NPM-ALK expression specifically in T-cells as has been carried out with limited success for various transgenic mice (17, 18, 23, 24). Regardless, the major caveat of using such a model system is the requirement to generate new mice from scratch for each experiment and hence the propensity for inter-experiment variability although one might argue that this might more closely resemble the heterogeneity of the human patient population.

5.2. Role of transgenic mouse models

Whilst chimeric models have been informative in proving the oncogenic potential of NPM-ALK *in vivo* they have also divulged the promiscuity of NPM-ALK suggesting that in order to develop a malignancy in mice resembling human ALCL, that expression must be targeted to the T-cell compartment. This is exemplified by findings in one of the first described transgenic NPM-ALK expressing mouse models whereby expression was targeted to the haemopoietic compartment by driving expression from the pan-haemopoietic *vav* promoter (18, 25). Utility of this promoter essentially enabled lineage screening for that preferentially transformed by NPM-ALK and in keeping with the large majority of chimeric model systems, the predominant phenotype was that of a B-cell lymphoma, in particular plasmacytoma (18). However, even with use of a T-cell specific promoter, namely the minimal CD4 promoter, plasmacytomas also developed in transgenic mice suggesting either some promoter leakage or cell fate decisions guided by NPM-ALK expression (17). This was also apparent in NPM-ALK transgenic mice whereby expression was directed by the T-cell specific CD2 promoter yet mice developed B-lymphoid malignancies (24). Regardless, the CD4/NPM-ALK transgenic line remains to date the best transgenic model for use in future studies, in particular the N16 line (which develops thymic lymphomas in greater than 90% of mice) and emphasises the importance of promoter choice when generating GEMMs (17).

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In all of these systems a true mimic of ALCL has remained elusive suggesting that either NPM-ALK must be expressed at a particular stage in T-cell ontogeny and/or that other events shape the final cell surface phenotype. In regard to the former, use of the T-cell specific lck proximal promoter also led to T-LBL development but with a disease so aggressive that a transgenic line could not be established again suggesting that expression at early stages in T-cell ontogeny is not conducive to the development of ALCL (23). Whereas in evidence of the latter scenario, ALCL has been associated with infectious agents varying from insect bites (ALK positive) to breast implants (ALK negative) suggestive of the aetiology of this disease and consistent with a mature T-cell origin (26, 27). Therefore, future efforts might need to concentrate on directing NPM-ALK expression to mature post-thymic T-cells and examining the effects of T-cell stimulation on lymphoma development. However, mice are not men and what can happen in mice does not necessarily correlate with what actually happens in humans although mature peripheral T-cell lymphomas in mice expressing NPM-ALK has been achieved following elimination of clonal competition: Newrzela *et al* recently reported that chimeric mice in which NPM-ALK is expressed in mature T-cells isolated from the spleens and lymph nodes of the T-cell receptor (TCR) transgenic mouse line OTI, before implantation into recipient mice, developed mature peripheral nodal T-cell lymphomas whereas mature T-cells from wild-type mice (also expressing NPM-ALK) or NPM-ALK expressing OTI T-cells co-transplanted with polyclonal wild-type T-cells could not. These data suggest that the polyclonal nature of wild-type T-cells is not permissive for lymphoma development whereas the OTI-derived T-cells expressing a clonal TCR (specifically recognising ovalbumin peptides) are (28). These data suggest that mature T-cells can be transformed by NPM-ALK to develop a disease more closely mimicking ALCL in keeping with the *in vitro* studies of Zhang *et al* (7).

Based on this literature, it clearly appears that essentially all chimeric/transgenic mouse models recapitulate only some features of human ALCL malignancies and that none of them precisely mimic the human disease. This statement could be explained first by the fact that the human ALCL stem cell has not yet been identified, thus making it difficult to identify the specific stage of T-cell ontogeny to genetically manipulate to reproduce ALCL; second the choice of promoter for expressing the ALK oncogene has been problematic for several research groups and has resulted in the diverse B-cell or immature T-cell lymphomas, as described above; third, ectopic expression of NPM-ALK does not use the endogenous promoter, is initiated from randomly integrated sites, and leaves intact both copies of each translocation participating gene (the one encoding ALK and the one encoding the dimerization partner). Thus, the tumour level of expression of the fusion protein and the heterozygosity of the translocation participating genes are not reproduced. Finally, chimeric models also suffer from intra-experimental variability and the requirement to generate a new set of mice with each experiment. We are now able to circumvent those caveats as explained below.

6. INVESTIGATING NOVEL THERAPEUTIC TARGETS AND FUTURE TREATMENT STRATEGIES

6.1. Genetically Engineered Mouse Models (GEMMs)

Tumours addicted to defined oncogenic events provide excellent model systems to investigate the effects of inhibiting said activity towards the therapy of the associated disease. Given the precedent set by Bcr-Abl and Imatinib, it was obvious that therapies targeting NPM-ALK might provide future therapeutic opportunities. A variety of *in vitro* studies, inhibiting NPM-ALK expression and activity through use of reagents as diverse as siRNA to small molecule inhibitors, have proven this potential and transgenic murine models have provided further evidence (reviewed extensively elsewhere). In particular, a transgenic model for ALK lymphomagenesis with inducible gene expression provided proof of principle (29). George Delsol's group used the tetracycline system (Tet OFF version) to conditionally express the ALK oncogene in the hematopoietic lineage, using the Eμ promoter previously reported in other systems to give rise to either B or T cell lymphoma (29-32). Not surprisingly, the preferential transformation of B-cells was observed in fitting with previous GEMMs as described above; tumours were blocked at the pro-B/pre-B-1 stage of differentiation, arising within a three-week latency but importantly were reversible upon doxycycline administration (and hence ALK silencing), thus confirming the role of the ALK oncogene in tumour maintenance and supportive of the use of ALK inhibitors as therapeutics (29).

Other studies have utilised GEMMs to illustrate therapeutic strategies in principle. Of note, a study by Chiarle *et al* showed that by back-crossing the CD4/NPM-ALK line to STAT3 knockout mice that the tumour incidence was severely reduced suggesting STAT3 as a therapeutic target although these findings remain to be translated to the clinic (19). Another noteworthy study that has successfully translated to clinical application from findings in murine models is the utility of PDGFR inhibitors, specifically Imatinib (33). In this study a human patient with ALCL refractory to existing available therapies was successfully treated with Imatinib and remains in complete clinical remission (personal communication Prof. L Kenner and (34)). Refined GEMMs more closely mimicking ALCL may provide useful in the future for assessing the efficacy of ALK inhibitors and other novel reagents. However, a number of xenograft systems have also proven useful in this regard, mostly cell line xenografts but increasingly PDX as well.

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6.2. Cell line xenografts

Engraftment of the different cell models described above into immune-compromised mice were and still are widely performed for two main purposes: Demonstration of the oncogenic properties of the cell models as well as the ease and rapidity of generation of an *in vivo* model to test the efficiencies of ALK cancer therapeutics. However, these model systems are limited by the sub-cutaneous location of tumours as well as the lack of a *bona fide* tumour microenvironment devoid of any inflammatory component. Regardless, they have been used extensively to examine the therapeutic potential of small molecule inhibitors including not only ALK inhibitors but also those affecting pathways activated by NPM-ALK (35-40). Traditionally, a xenograft of just one cell line representing the entire disease spectrum is employed as proof of principle that the novel compound will have therapeutic efficacy. However, it is well known that many drugs fail early in clinical trials because of the heterogeneity of the patient population. Hence, there has been a gradual move towards PDX as a model system although in the case of ALCL this is slightly hampered by the relative rarity of this disease.

6.3. Patient-derived xenografts (PDX)

PDX are based on the "culturing", maintenance and propagation of tumours directly isolated from the patient in immune-compromised mice. PDX models present the notable advantage to both recapitulate expression and genetic profiles of primary tumours as well as to respond to known therapeutics in a similar fashion to the primary tumour (41). The first reports of PDX models of ALCL were reported in 1995 and 1999 by Marshall Kadin's group (42, 43). Their studies showed that ALCL PDX closely resemble the primary tumour in histopathology and could be used to test the efficacy of CD30 targeted immunotherapy (44). These initial studies have been repeated to assess the efficacy of other therapies for example ALK inhibitors and undoubtedly more will follow (43, 45). However, we must point out here that the latency period to develop tumours in PDX can vary between 2 and 12 months and that the most successful tumour engraftments originate classically from high grade tumours at late stages of tumour development, which is not ideal for studying early stages of tumorigenesis. Thus, PDX models are good tools for drug screening and preclinical studies, but less so for basic cancer research. In addition they do not fully recapitulate the patient tumour in that the mouse host is immune-deficient and the tumours are often sub-cutaneous rather than nodal as is the case for ALCL.

7. THE FUTURE OF ALCL MODEL SYSTEMS

7.1. Refinement of existing model systems

Whilst as a community we have made considerable progress in understanding the role of NPM-ALK and other ALK oncogenes towards the pathogenesis of cancer there still remain many unanswered questions. Of note, the natural progression of ALK-induced ALCL has not been elucidated and the question remains as to why in human patients we observe a peripheral T-cell lymphoma that has proven difficult to model *in vivo*. There are many possibilities, some more obvious than others but at its very heart we have not yet developed a mouse in which the exact genetic events are copied, in particular with the generation of the t(2;5)(p23;q35) is not only the gain of the *NPM-ALK* fusion gene but also a reduction of *ALK* and *NPM* gene dosage to 1 copy. Given that ALK appears to be a neural specific protein, this reduction in gene dosage is not likely to have an effect on lymphoid malignancies but given the ubiquitous expression and function of NPM this may be of significance (46-48). However, as a counter-argument, the presence of a large variety of ALK fusion proteins suggests that *NPM* heterozygosity is not of importance and this was backed up in an *in vitro* study whereby the importance of NPM was resigned to its ability to induce dimerization and hence autophosphorylation of NPM-ALK (48). In addition, a murine study in which *NPM-ALK* transgenic mice were back-crossed onto an *NPM* heterozygous background which did not result in any change in disease latency supports this theory although one might argue that the NPM-ALK GEMM of choice (CD2/NPM-ALK) did not sufficiently mimic human ALCL (49, 50). Hence, this remains to be explored further perhaps using model systems as detailed below.

Equally compelling is our lack of knowledge as to the true cell of origin for this malignancy and the events that shape the presenting disease phenotype, i.e. mature T-cells in some cases appearing of a cytotoxic phenotype and in others a helper CD4-expressing T-cell, Treg, Th17 or 'null' cell (9, 51, 52). However, many of these features that presume this array of T-cell subset phenotypes have been attributed to NPM-ALK activity perhaps independent of the cell of origin. For example, it has been shown that NPM-ALK can induce cellular production of cytotoxic proteins, IL17 and FoxP3 expression (51-53). Hence, it is possible that the cell of origin is a common precursor T-cell and in evidence a recent study has indicated that ALCL cancer stem cells (CSC) may well have origins in early thymic progenitors (54). If this is the case, then it is clear, at least in mouse models that other events must dictate the disease phenotype as expression of NPM-ALK in immature T-cells in mice leads predominantly to lymphoblastic lymphoma as discussed above, whereas expression in mature T-cells in the absence of polyclonal competition *in vivo* gives rise to peripheral T-cell lymphoma (28).

7.2. New technologies

Despite significant progress, a true murine mimic of ALCL, particularly a GEMM remains elusive but as technologies evolve as does our understanding of this disease, it is highly likely that model systems will improve to

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become more informative. Zinc-Finger Nucleases (ZFNs), Transcription Activator-Like Effector Nucleases (TALENs) and Clustered Regulatory Interspaced Short Palindromic Repeats (CRISPR) / CRISPR-associated (Cas) systems are currently redefining the boundaries of genome engineering (55). ZFNs and TALENs are engineered nucleases composed of sequence-specific DNA-binding domains fused to a non-specific DNA cleavage module (56). The DNA binding domain can be customized at will, thus allowing the recognition and subsequent cleavage of any DNA sequence. CRISPR/Cas are programmable RNA-guided DNA endonucleases, that have multiplexed gene disruption capabilities (57). The precise DNA double strand breaks induced by either one of these three DNA nucleases, then stimulate classical DNA repair mechanisms (NHEJ or HDR) (58). Thus, programmable DNA breaks could create, *de novo*, at the exact endogenous loci, a translocation-associated oncogene. Most importantly, proof of principle of this powerful technology (notably TALENs) in genome editing has been reported for the *de novo* creation of the NPM-ALK translocation in two different cell settings: Jurkat T cells and RPE-1 cells (retinal pigment epithelial cells) (59). Thus, it is tempting to speculate that *de novo* creation of the NPM-ALK oncogene either in its natural target cell, in human induced-pluripotent stem cells (iPS), or in mouse blastocysts, hold the promise to lead to the generation of the most accurate model for ALK-positive ALCL. It is equally possible that other established technologies such as the 'translocator mouse' system developed in the lab of Terry Rabbitts might provide a source of GEMM mimics of ALCL (60). Alternatively, other model systems might provide better mimics of ALCL, for example zebrafish models are more commonly in use for the study of tumorigenesis, more recently models of T-LBL have been described (61).

8. CONCLUSIONS

We believe a new era for ALCL personalized medicine is coming, taking advantage of both the generation and use of PDX and GEMM models (closely mimicking ALCL pathology) and their potential enrolment in co-clinical trials (62) (as previously reported for EML4-ALK NSCLC (63)). Indeed, this real-time "GEMMs-to-human" transition allows the concurrent integration of murine and human data, thereby allowing better, personalized and timely clinical decisions to be made (41). In the particular field of ALCL, like in other types of cancers, this will considerably facilitate and improve both drug development and patient health care management. Thus, in the coming years, it is likely that the use of ALCL mouse models will evolve from the classical study of the oncogenic ALK lymphomagenesis process to more clinically oriented applications.

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Abbreviations: ALCL, Anaplastic Large Cell Lymphoma; NPM, Nucleophosmin; ALK, Anaplastic Lymphoma Kinase; TCR, T-cell receptor; GEMMs, Genetically Engineered Mouse Models; PDX, Patient Derived Xenografts; CSC, Cancer Stem Cells; iPS, induced pluripotent stem cells; MOI, Multiplicity of Infection; T-LBL, T-Lymphoblastic Lymphoma; ZFN, Zinc Finger Nucleases; RPE-1, retinal pigment epithelial cells; TALEN, Transcription Factor Activator-Like Effector Nucleases; CRISPR, Clustered Interspaced Short Palindrome Repeats; cas, CRISPR-Associated Systems.

Key Words: ALCL, NPM-ALK, Mouse models, Cancer models, *In vitro* cancer models, Review

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