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Beyond NPM-ALK driven lymphomagenesis: alternative drivers in Anaplastic Large Cell Lymphoma

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ABSTRACT

PURPOSE OF THE REVIEW: Anaplastic Large Cell Lymphomas (ALCLs) are rare entities whose tumorigenic events have only been found in well-defined subsets. The categorization of additional molecular fingerprints is needed to advance our knowledge and to deliver successful therapies.

RECENT FINDINGS: The discovery of Anaplastic Lymphoma Kinase (ALK) fusions has provided the basis for the characterization of distinct subsets among ALCL patients. Although the oncogenic addiction of ALK signaling is proven, the tumorigenic contribution of co-activating lesions is still missing. As ALK- and ALK+ share common signatures, it is plausible that analogous mechanisms of transformation may be operating in both subsets, as confirmed by the dis-regulated activation of c-MYC, and the loss of Blimp-1 and p53/p63 axis. Nonetheless, recurrent genetic alterations for ALK- ALCL or refractory leukemic ALK+ ALCL are lacking. Moreover, although conventional chemotherapies (anthracycline-based) are most successful, i.e. in ALK+ ALCL patients, the implementation of ALK inhibitors or of anti-CD30 based treatments provides innovatites solutions, particularly in pediatric ALK+ ALCL and in chemorefractory/relapsed patients.

SUMMARY: The complete portrayal of the landscape of genetic alterations in ALCL will dictate the development of innovative chemotherapeutic and targeted therapies that will fit most with the molecular and clinical profiling of individual patients.

Key words:
Anaplastic Lymphoma Kinase, signaling pathways, kinases' inhibitors, lymphoid differentiation, mouse models.

KEY POINTS:

- Anaplastic Large Cell Lymphoma are an heterogeneous group of lymphoma some of which carry a restricted number of genetic defects mainly involving the Anaplastic Lymphoma Kinase (ALK+ ALCL) o less frequently display alternative translocations [t(2;x)(p23;x), t(6;7)(p25.3;q32.3) and inv(3)(q26q28) leading to TBL1XR1/TP63, etc.]. Nonetheless, specific lesions are still lacking for many ALCLs.
- The precise relationship and origin of ALCL remain unclear. Although ALK+ and ALK- share a set of genes and similar phenotypes, they are considered distinct groups with unique clinical features. However, it is unknown whether clinical differences and responses to conventional therapies may simply be related to different clinical stages and/or unique genomic lesions.
- Bioinformatics algorithms have identified several ALCL signaling classifiers demonstrating the preferential expression of a restricted number of pathways. The recognition of "common hubs", which can be targeted by selective inhibitors, represents a viable strategy for future therapeutic protocols.
- Taking advantage of *in vitro* and *in vivo* models, several groups have shown that the transforming properties of ALK fusions involve a plethora of alternative modules capable to regulate intrinsic (i.e. cytoskeleton, cell growth, etc.) and/or extrinsic (cell matrix invasion, tumor-host relationships, etc.) modalities.
- The identification of the driving lesion of ALCL will require the construction of international networks capable to synergize their activities and to construct large and clinically annotate tissue libraries. The collection of viable tissues will facilitate the generation of batteries of "Patient Derived Tumorgrafts".

INTRODUCTION

Peripheral T-cell lymphoma (PTCL) are a heterogeneous group of tumors derived from post-thymic elements including leukemic/disseminated, nodal and extranodal diseases [1,2,3]. As orphan diseases (12 to 15% of all non-Hodgkin's lymphoma [NHL] in Western populations) [1,2,3,4], they include entities displaying a great variability in clinical, morphological, immunophenotypic, cytogenetic and molecular features. First described in 1985 [5], Anaplastic Large Cell Lymphoma (ALCL) of either adults (2-8% of NHL) or children (15-30% of NHL), nowadays correspond to specific subtypes of systemic peripheral T-cell lymphoma [1]. The presence of Anaplastic Lymphoma Kinase (ALK) gene fusions has provided the criteria for a new WHO classification, which contemplates a novel entity (i.e. ALCL ALK+) and proposes a provisional one, including ALCL patients, who lack ALK translocations (i.e. ALCL ALK-). Because of genetic, immunophenotypic, and clinical differences, cutaneous ALCLs (cALCL) are considered as a completely distinct subset a part from its systemic counterparts. Systemic ALCL share cytological, immunophenotypic and molecular features. However, ALK- ALCL patients have poorer performance status, more often B symptoms [6], and an overall survival (OS) rate of 36% versus 20% of PTCL-NOS patients. This suggests unique driving defects, with high oncogenic penetrance. In contrast, ALK+ ALCL have a more favorable clinical course [4], though ALK+ ALCL with an aggressive behavior could be encountered in the clinical practice [7]. It remains uncertain whether the molecular lesion(s) and/or other features determine the clinical course of ALCL patients. In fact, once patients are normalized by clinical parameters, ALK- and ALK+ ALCL display analogous prognosis (failure-free survival [FFS] and OS) [6]. Considering that ALK+ ALCL have a less complex karyotype [8,9,10,11], it plausible that ALK fusions are critical actors and that tumor progression is due to somatic mutations (minimal deletions, activating somatic mutations, etc.) disrupting the function of a limited set of genes. In contrast, the transformation of ALK- ALCL might require the consolidation/acquisition of many genetic defects that rapidly lead to systemic and more aggressive phenotype. This is in agreement suggested with their higher and heterogeneous karyotypes [8,9,10,11]. Nonetheless, the driving lesion(s) of ALK- ALCL are still to be identified, and co-drivers are lacking for both ALCL subgroups.

The lack of representative cell lines or animal models has definitively contributed in impairing our knowledge of mature T-cell lymphoma. Ultimately, this has jeopardized the design of successful therapies and the upgrade of clinical programs, particularly in patients with poor outcome (ALK- ALCL and PTCL-NOS). The recognition of the tumorigenic defects of PTCL is

expected to provide patient specific "molecular fingerprints" and thus more suitable tailored therapies.

ONCOGENIC SIGNALING OF ALK FUSIONS

Chromosomal translocations of the *ALK* gene are documented in many ALCL, although the percentage of ALK+ ALCL varies, as a result of the inclusion criteria of the ALK- ALCL. In absence of strong classifier(s) (like ALK), the distinction between ALK- ALCL, CD30 PTCL-NOS and some enteropathy associated T-cell lymphoma represents a diagnostic challenge.

ALK gene encodes a 210kDa tyrosine kinase receptor (CD247) belonging to the insulin growth factor receptor superfamily. Its expression is largely limited to the nervous system during embryogenesis and to focal areas of the adult brain [12]. Although the physiological role of ALK in mammals is unknown, it is involved in neuronal differentiation [13] and ALK activating mutations have been found in familial and sporadic neuroblastomas [14].

The breakpoints of ALK chimera invariably occur within the intron placed between the exons 19 and 20 (NM_004304.3). Thus exons coding for the intracytoplasmic domain of ALK (exons 20–29) are then juxtaposed to different partners [15,16]. Seventy-80% of ALK+ ALCL harbor the t(2;5)(p23;q35) translocation (NPM–ALK chimera). The intracellular distribution of the fusions is due to the structure/function of ALK partners, enforcing either to nuclear/cytoplasmic, or cytoplasmic and in rare cases juxta-membranous localization. Virtually all partners (with the exclusion of MYH9-ALK) provide dimerization domains, leading to the homo/heterodimerization of the fusions and to constitutive activation of the kinase [16,17]. Conventional genomic approaches, and more recently next generation sequencing (NGS) have shown alternative ALK translocations in many types of human cancers, e.i. lung tumors [18,19].

ALCL display additional alterations involving many chromosomes [8,9,10,20], however frequent common/discrete secondary lesions are rare. Two translocations were reported in ALK-ALCL, involving the *DUSP22* gene, which is juxtaposed to the *FRA7H* fragile site, or to the gene coding for *IRF4* [21]. Boi et al. have recently shown that several ALCL display recurrent deletions affecting 17p13.3-p12 (25%) region, in which *TP53* gene is located, and 6q21 (19%) encompassing *PRDM1* and *ATG5* genes [11]. Finally, Vaismatzis et al. have recently described a set of genomic defects in DLBCL and PTCL/ALCL encoding fusion proteins homologous to ΔNp63, a dominant-negative p63 isoform that inhibits the p53 pathway [22].

ALK chimera were originally proven to be oncogenic *in vivo* [23] and these data were largely confirmed in *in vitro* models [24] and then in genetically modified animals [25,26]. Understanding how ALK signals acts and defining the mechanisms responsible for its

deregulation is critical for dissecting the mechanisms, which mediate ALK cellular transformation and provide the basis for *rationale* therapeutic approaches. By a large array of methods, it is now know that ALK fusions and in particular the NPM-ALK chimera interact with a plethora of molecules and elicit many pathways. These include the RAS/Erk, PLC-γ, PI3K, and Jak/Signal Transducers and Activators of Transcription (STAT), capable to control individually or in association, cell proliferation, survival, and cytoskeletal properties [19].

The activation of RAS/Erk pathway provides positive signals regulating cell growth and the inhibition of MEK (AZD6244 or shRNA) leads to cell cycle arrest, without significant changes in cell viability (Crescenzo R, personal communication). Similarly, NPM-ALK can down-modulate, via PI3K-AKT, the inhibitory action of FOXO3a, upregulating Cyclin D2 and down-regulating p27, and providing positive growth signals.

We and other groups have shown that the neoplastic phenotype of NPM-ALK is largely mediated by the STAT3. This enforces the transcription of a surplus of genes (coding and non coding), promoting cell growth and survival. In shRNA-based knockdown experiments, Piva et al. have demonstrated that several genes are directly regulated by STAT3, which display canonical STAT3 binding sites within their regulatory regions (Piva R, personal communication). Among them we mention CD30, granzyme, perforin, IL1RAP and IL2RA. From a diagnostic point of view, CD30, granzyme, perforin are known to be preferentially expressed by ALCL cells, and are commonly used in algorithms encompassing the differential diagnosis of different PTCL entities. Their transcription requires phosphorylated STAT3 complexes, which often include CEBPb and AP-1 transcription factors. Notably AP-1 members play an important role in ALK mediated transformation controlling tumor growth and positive host signals, via PDGF [27] (Fig. 1).

Zhang and coworkers have recently elucidated additional features of STAT3, demonstrating a STAT3-positive regulation of ICOS [28]. The same group had previously shown that PDL-1 expression is also regulated by STAT3 [29]. Collectively, these data demonstrate that ALCL cells engage ICOS to gain a growth advantage, and PDL-1 as novel mechanism of tumor escape, modulating the host responses. The overexpression of IL-21 [30] and deregulation of TNF/Fas/TNF [31] can also contribute to ALK tumorigenic phenotype, favoring the success of ALCL cells and overcoming host defenses. Finally, ALK signaling controls HiF1α, a factor that impacts directly in the neo-angiogenesis and provides a positive growth advantage to the lymphoma cells [32,33] (Fig. 1).

Lastly, STAT3 itself can directly or via downstream mediators down-regulate the transcription of many genes. Approximately 60% of modulated STAT3 genes are repressed after shRNA KD.

Interestingly, Zhang et al have recently reported that STAT3, engaging the IL-2Rγ promoter, enhances the binding of DNA methyltransferases (DNMTs), leading ultimately to the transcriptional repression of IL-2Rγ gene. The knockdown of IL-2Rγ expression contributes to the neoplastic phenotype, as demonstrated by its forced/expression that leads to the loss of NPM-ALK protein expression, and then apoptosis. Ultimately, STAT3 down regulates T-cell associated molecules controlling T-cell identity of ALCL cells. In this context NPM-ALK provides signals capable to bypass TCR mediated activation [34,35] (Fig. 1).

STAT3 can similarly regulate the expression of several miRNA clusters (Spaccarotella E, personal communication) including the miRNA17-92 [36], known to have a role in human cancers. In ALCL, the miRNA17-92 overexpression overcomes in part the loss of STAT3 in an shRNA STAT3 inducible ALK+ ALCL model. More importantly, primary ALK+ ALCL display higher miRNA17-92 levels [36] compared to ALK- ALCL and cutaneous T-cell lymphoma and the usage of STAT3 inhibitors leading to the down-regulation of this cluster could represent an attractive strategy for the treatment of ALCL lymphoma (Lin C, personal communication).

The ability to successfully migrate and invade distant tissues contributes to the neoplastic phenotype, impairing clinical responses and long remissions. ALK signaling can efficiently module the cytoskeleton and promote invasion. The data reported by Ambrogio et al. [37] have recently been confirmed [38]. Dupuis-Coronas et al. have also shown that ALK, modulating the activity of PIKfyve, enhances the invasive capacities of NPM-ALK cells and their capacity to degrade the extracellular matrix [39]. Invasion of ALK+ ALCL cells is also modulated by the axis ALK-STAT3-Twist1 [40] (Fig. 1).

In conclusion, it is evident that the tumorigenic properties of ALK signaling are more complex that originally proposed, confirming that ALK is a powerful kinase capable to provide a complete and broad oncogenic addiction. These properties make ALK an excellent therapeutic target.

ALKR AND ALKS ANAPLASTIC LARGE CELL LYMPHOMAS: TWO SIDES OF THE SAME COIN?

The debate on distinct entities among PTCLs remains open. Novel hypotheses are emerging on the origin and relationship of different PTCL entities. The concept that ALK- ALCL should be lumped within PTCL-NOS has been recently sponsored. Alternatively, a scenario in which all ALCL are incorporated in a single group, irrespectively of the ALK expression has been contemplated. This level of uncertainty is corroborated by the fact that, once ALCL patients are stratified by stage, IPI etc. either groups display similar characteristics. In this landscape, CD30+

PTCL represent a puzzling/confounding group [41]. Their precise definition is critical and sometime questionable. Immunophenotypically, they express weak/partial CD30, and in same cases CD15 [42,43]. Cytologically display a certain monomorphism and they often have a functional TCR signaling (NFATc positive etc.) [44,45]. Clinically, CD30+ PTCL share a more aggressive clinical course, justifying their distinction and a closer relationship to PTCL-NOS. We strongly believe that these uncertainties will be solved only when distinct molecular defects will be discovered in different PTCLs.

Another similar confusing topic regards the ALCL origin, and their putative normal counterpart elements. Several hypotheses have been proposed, taking in account their expression profile and unique immune-phenotype. The expression of perforin, T1A1 and granzyme has been interpreted as a specific fingerprint, supporting the idea that ALCL may derive from cytotoxic T-lymphocytes. Alternatively, we speculate that the phenotype of ALCL may rather be the result of the deregulated expression of unique pathways and/or specific defects, which impose unique/fixed profiles. It is known that transcription factors can play a critical role in T-cell differentiation and once constitutively activated can undermine physiological programs and rerouted their development. Based on this assumption, we could speculate that the constitutive activation of STAT3 might be responsible for the cytotoxic phenotype of ALK+ ALCL cells, even in cells that were committed to different lineages and/or function. This leaves the open question, why ALK- ALCL display a cytotoxic phenotype? To solve this question, we have analyzed a large cohort of ALCL samples and found that a subset of ALK- ALCL clearly shares a STAT3 expression profile and detectable nuclear pSTAT3. Moreover, both ALK+ and ALK- ALCL reveal signatures, linked to the activation of c-MYC, NOTCH-1, or NFkB, and RAS/ERK, suggesting the existence of upstream activators. Interestingly, it now evident that several ALCL co-share overlapping signatures suggesting multiple activating defects or alternatively the presence of unique lesions capable, like ALK fusions, to efficiently and concomitantly fire multiple pathways. Search of ALCL pathogenetic lesions is under evaluation and it is predicted that new information will be available soon (Fig. 2).

CAN WE USE PRECISION MEDICINE DATA TO IMPROVE THERAPUTIC COMPLIANCE?

The definition of the molecular fingerprints of neoplasms is now possible through the implementation of the impressive technologies. The NGS platforms are currently entering the clinical arena and it is plausible that, once interconnected and clinical based networks of laboratories, many patients will have individualized molecular identikits. Nonetheless, caveats

on the tumorigenic contribution of individual lesions and their functional role in the maintenance of the neoplastic phenotypes remain untouched. This are a critical issues, which should be added to the overwhelming capacity of tumor cells to adapt rapidly to the environment and to stress imposed by drugs and host changes. Thus, the search the "magic bullet" may fail. Instead, the association of multiple "smart" compounds could provide higher response rates and overcome resistance. Since the cost for a novel drug is around 1billion and requires approximately 12-15 years, we need to overcome impairing inefficiencies. It is agreed that many improvements in discovery programs need to be rapidly put in place, meliorating company inefficiencies (structural and operation), selection of viable targets, defining good therapeutic biopredictors, innovative technologies, more efficient and reliable screening tests and faster and less expensive clinical tracks in molecularly defined and/or naïve patients.

While pharmaceutical companies are reshaping their pipelines, a small number of drugs is successfully introduced into clinics. This ineffective result is seemingly related to update pre-clinical models, heavily relaying on *in vitro* models and "xenografts mouse platforms". Indeed, the most frequently used cell lines poorly represent human tumors [46]. This has encouraged many institutions and drug industry to acquire large library of cell lines, which can be interrogated with HTP platforms (NSG, phosphomapping etc.). The hope is to define better criteria and relationships between the genome and responses to therapies. The hope is of predicting more reliable clinical responses and dissecting responders and refractory patients. But, cell lines lack the host and its regulatory networks, have undergone ferocious in vitro selections and do not represent tumor heterogeneity.

To solve some of these issues, implants of fresh primary neoplasms are frequently introduced in severally-immunocompromised mice [47]. The generation of individualized cancer models represents an unprecedented opportunity to test battery of drugs for each individual and provide personalized oncology programs. However, these strategies need to be linked to defined genetic defects. Only combining HTP and innovative models, we can deliver a list of targetable lesions, which once validated in animals, should provide reasonable expectations. Since the successful growth of tumorgraft implants may require long period of time, new technologies interrogating the functional network in cancer and the efficacy of chemical libraries *in vitro* may provide alternative routes for the execution of pre-clinical trials *in vivo*. Our group has recently embarked in such a program and generated a battery of ALCL "Patients Derived Tumorgrafts" (PDT) [48]. These retain the immunophenotypic, genomic features of their corresponding primary tumors and display responses to conventional and innovative protocols that closely mimic those seen in donor patients. Their molecular characterization has demonstrated the presence of unique

genomic defects and allowed to discover new pathogenetic translocations and activating somatic mutations. The definition of a molecular identikit in PDTs will provide not only patients' fingerprints but also models to test the efficacy of selected drugs targeting hypothetical tumorigenic defects in each patient in vivo.

CONCLUSION

Little is yet known of mechanisms leading to T-cell lymphomagenesis. Nonetheless, the systematic usage of high throughput platforms has recently demonstrated that recurrent defects may be present in specific subsets of PTCLs. Although ALKþ ALCL and ALK- ALCL display heterogeneous complex karyotypes, they share common expression signa- tures and dysregulated signalling pathways. The use of NGS approaches will be instrumental for the more complete discovery of mechanisms driving the pathogenesis of ALCL. New molecular lesions, even in small subgroups of patients, will provide objective diagnostic criteria and the bases for 'intelligent' therapies, to be first validated in the most informative preclinical models (i.e. PDT and so on)

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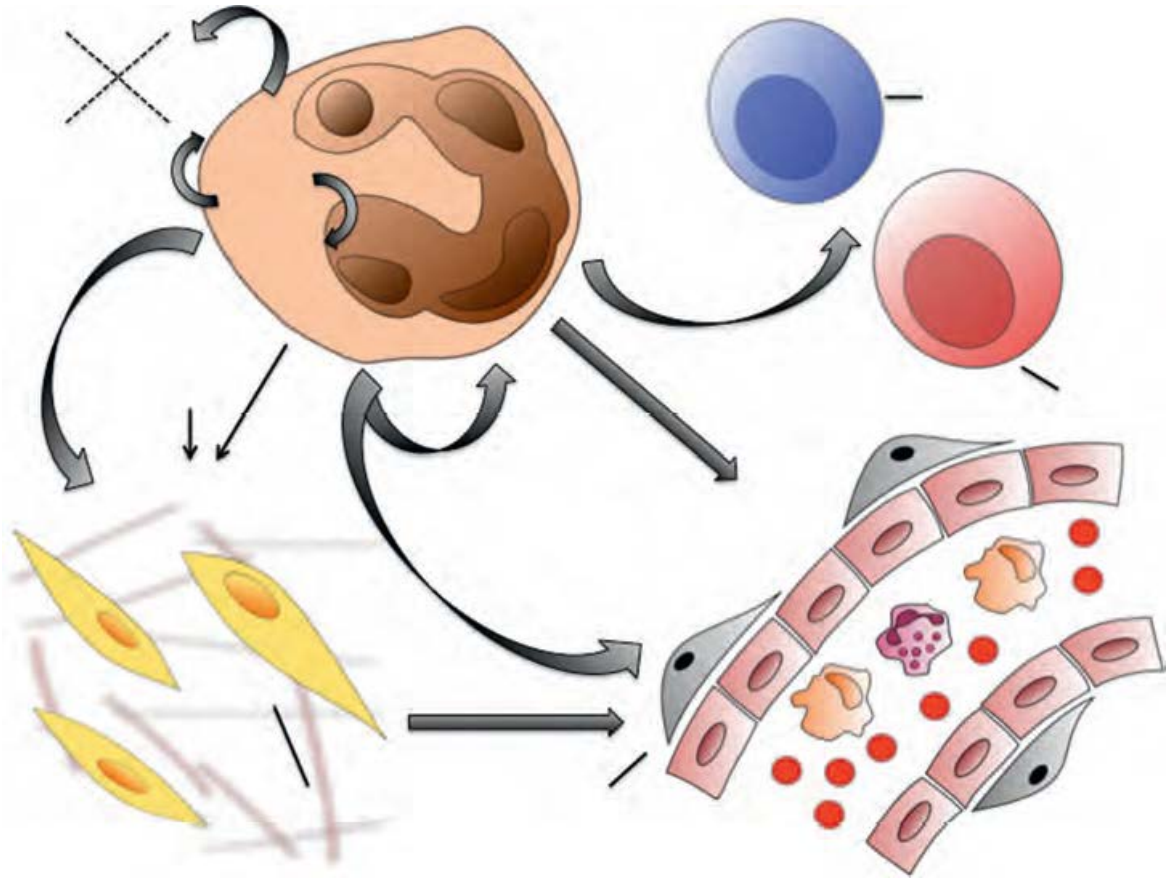

FIGURE 1. Anaplastic large cell lymphoma cells dysregulate and control the host environment. The oncogenic drivers of ALCL reroute intrinsic pathways leading to a self-autonomous cell growth as well as overcome host confinements, modulating immuno-responses of regulatory and effector T-cells. This is accomplished through alternative mechanisms including antigen-camouflaging and lymphokines production obliterating immune surveillance (i.e. TNF/FasR, PD-L1/PD-L1R). Through cytoskeleton changes and production of pro-invasive mechanisms, tumour cells have the ability to migrate locally and disseminate to distant organs. Production of pro-angiogenetic factors stimulates vessel formation providing the necessary growth support for tumour survival. The JAK – STAT signaling pathway represents a master culprit modulating gene expression transcription of critical players. ALCL, anaplastic large cell lymphoma; CDC42, cell division control protein 42 homolog; FasR, FAS receptor; HIF1a, hypoxia-inducible factor 1 alpha; IL, interleukin; JAK, Janus kinase; MMPs, matrix metalloproteinases; PDGF, platelet-derived growth factor; PD-L1, programmed cell death 1 ligand 1; PIKfyve, finger-containing phosphoinositide kinase; pSTAT3, phosphorylated signal transducer and activator of transcription 3; TNF, tumour necrosis factor; TWIST1, twist-related protein 1; VEGF, vascular endothelial growth factor.

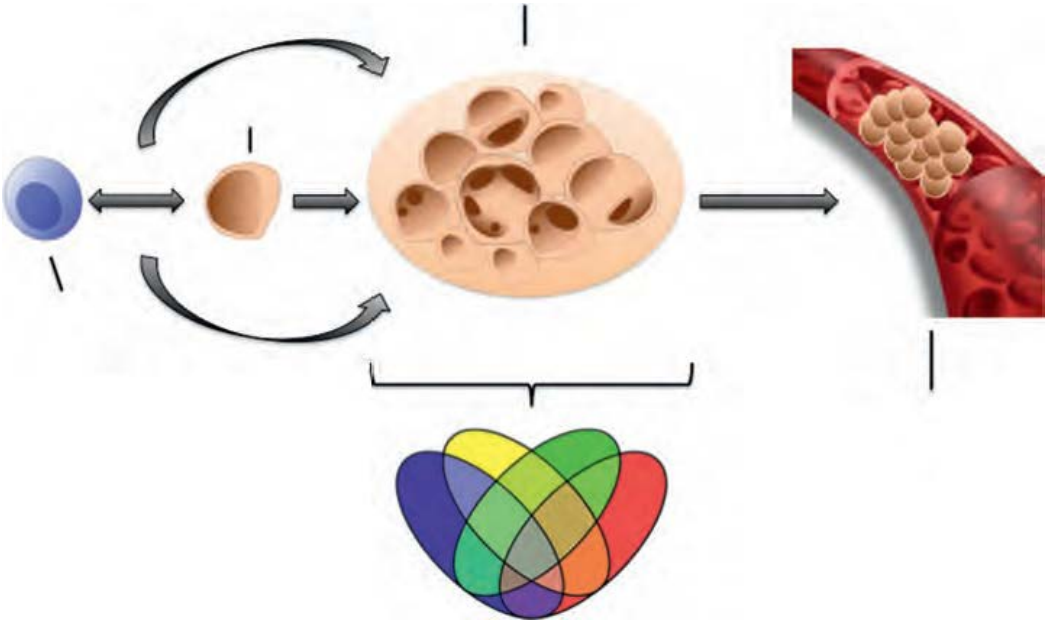

FIGURE 2. Tumourigenic model for anaplastic large cell lymphoma transformation. ALCL may derive from a common stem cell precursor or alternatively from partially committed T element(s). Through the acquisition of powerful oncogenetic drivers, ALCL cells acquire unique phenotypes and display restricted signaling pathways. The progressive acquisition of selected genetic defects (loss of TP53, TP63, BLIMP1 and constitutive activation of c-MYC) is eventually responsible for tumour progression and more aggressive clinical behaviours. ALCL, anaplastic large cell lymphoma; ALK, anaplastic lymphoma kinase; IRF4, interferon regulatory factor 4; PRDM1, PR domain zinc finger protein 1; STAT3, signal transducer and activator of transcription; TKR, tyrosine kinase receptor; TP, tumour protein.

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