ALK signaling and target therapy in anaplastic large cell lymphoma

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The discovery by Morris et al. (1994) of the genes contributing to the t(2;5)(p23;q35) translocation has laid the foundation for a molecular based recognition of anaplastic large cell lymphoma and highlighted the need for a further stratification of T-cell neoplasia. Likewise the detection of anaplastic lymphoma kinase (ALK) genetic lesions among many human cancers has defined unique subsets of cancer patients, providing new opportunities for innovative therapeutic interventions. The objective of this review is to appraise the molecular mechanisms driving ALK-mediated transformation, and to maintain the neoplastic phenotype. The understanding of these events will allow the design and implementation of novel tailored strategies for a well-defined subset of cancer patients.

Keywords: anaplastic large cell lymphoma, chimeric fusion proteins, signaling pathways, molecular targeted therapy, anaplastic lymphoma kinase

INTRODUCTION

The concept of anaplastic large cell lymphoma (ALCLs) has changed over the years as a result of a fruitful stream of new information and novel understanding about the cell of origin, biology, genetics, and clinical features of these neoplasms. Most importantly, the discovery of anaplastic lymphoma kinase (ALK) fusion chimera, among ALCLs, has provided the basis for the definition of a unique subgroup among peripheral T-cell lymphoma (PTCL) and set the stage for the identification of new entities, some of which still remain provisional within the current WHO Classification of tumors of hematopoietic and lymphoid tissues.

Indeed, the discovery of the Nucleophosmin (NPM)–ALK fusion, in many but not entirely systemic ALCL, have led to the recognition that ALCLs are biologically and clinically heterogeneous. In fact, it has likewise become evident that ALK+ ALCLs, most commonly seen in children and young adults, display a more favorable prognosis. On the contrary, ALK− ALCLs have a more aggressive clinical course and could represent a distinct morphologic variant among PTCL, not otherwise specified, NOS (PTCL–NOS; Delsol et al., 2008).

Although the molecular stratification of ALCLs has provided objective criteria for precise diagnoses, many issues remain open. As a matter of fact, we do not know the mechanisms leading to the transformation of ALK− ALCLs, including those involving the skin primarily (cALCL), or whether various entities among ALCLs somehow share common pathogenetic features and if putative relationship(s) with other T-cell lymphoma may exist. These issues are critical for the design efficient therapies, tailored to unique subsets, targeting individual biological and genetics defects.

The main objective of this review is to undertake a systematic appraisal of ALK deregulation in human cancers and in particular in ALCLs, with the intent of discussing novel targeted therapies and/or personalized therapeutic modalities.

PERIPHERAL T-CELL LYMPHOMA

T-non-Hodgkin lymphoma (NHLs) include a large number of neoplasia, most likely representing the neoplastic counterpart of functionally distinct T-cells, transformed by unique but still largely unknown genetic defects. Among those derived from mature T-lymphocytes, two subsets have been described, known otherwise as PTCL specified and NOS. These lymphoma are overall quite rare, ranging from 12 to 15% among all NHL in Western populations (**Table 1**; Vose et al., 2008). Many of them display a great variability in their clinical, morphological, immunopheno-typic, cytogenetics, and molecular features. PTCL–NOS is the

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Table 1 | Geographic distribution of anaplastic large cell lymphoma compared to peripheral T-cell lymphoma, not-otherwise specified (PTCL–NOS).

	PTCL	
	PTCL–NOS	ALCL
GEOGRAPHIC DISTRIBUTION (%)		
USA	3	2
Canada	1	3
South Africa	8	3
England	8	2
Germany	4	1
France	4	3
Switzerland	6	0
Hong Kong	10	3

Table 2 | Clinical features of anaplastic large cell lymphoma compared to peripheral T-cell lymphoma, not-otherwise specified (PTCL–NOS).

		Disease	
		PTCL–NOS	ALCL
DESCRIPTIONS			
No. of cases		2,148	1,296
Mean age at diagnosis ±SD		58.4 ± 19.0	50.5 ± 22.0
5-Year RS (%)		38.7	54.8
DEMOGRAPHIC DISTRIBUTION			
Non-Hisp W	No. (%)	826 (38.5)	524 (40.4)
	Age ± SD	58.8 ± 18.0	51.3 ± 20.9
Hisp W	No. (%)	122 (5.7)	121 (9.3)
	Age ± SD	46.8 ± 18.8	40.9 ± 23.7
Black	No. (%)	170 (7.9)	97 (7.5)
	Age ± SD	51.5 ± 17.0	46.9 ± 17.0
AI/API	No. (%)	139 (6.5)	49 (3.8)
	Age ± SD	59.0 ± 19.6	43.2 ± 23.1

Non-Hisp W indicates Non-Spanish–Hispanic–Latino White; Hisp W indicates Spanish–Hispanic–Latino White; AI/API indicates American Indian/AK Native and Asian/Pacific Islander.

most common (30–40%), followed by the angioimmunoblastic (AITL) and ALCLs. ALCLs occur in ∼2–8% of NHL in adults and represent 15–30% of all high-grade lymphoma in children.

Systemic ALCLs are classified as *de novo* and secondary (anaplastic transformation of other lymphoma). *De novo* ALK+ ALCLs are clinically aggressive lymphoma that frequently occur within the first three decades of life, with a typical male predominance. They often present as a stage III–IV disease, with systemic symptoms, and extranodal involvement (60%; Falini et al., 1999; Stein et al., 2000). ALK− ALCLs arise in older patients (most commonly at sixth decade of life), with a slight predominance in males (Savage et al., 2008), and high International prognostic index (IPI) scores (Tilly et al., 1997; see **Table 2**). Extranodal involvement is relatively common with cutaneous, hepatic, or gastrointestinal sites most frequently engaged. ALK− ALCLs, when compared to ALK+ ALCLs, display a worse clinical presentation and outcome, meanwhile they have a relatively better overall survival rate than PTCL–NOS (36 versus 20%, respectively; Vose et al., 2008). The more favorable clinical outcome of the ALK+ ALCLs (Savage et al., 2008) has been associated to the age of occurrence and to the driving molecular defects, in particular to ALK fusions. When ALCL patients are however studied taking into account the age and/or other clinical parameters, ALK+ and ALK− ALCLs display analogous clinical features, suggesting that relevant clinical factors may have critical implications on their outcome. Nevertheless, it is unclear whether these clinical features are due to and/or associated to restricted defects occurring at different ages and/or accumulate overtime. Besides the well-defined primary chromosomal translocations, ALK+ ALCLs carry frequent secondary chromosomal imbalances, although their complex karyotype is substantially less complex than those observed in ALK− ALCL, suggesting that the more heterogeneous genetic setting of the latter group may be associated to a worse clinical outcome. If this hypothesis is correct, one could postulate that the ectopic activation of ALK provides dominant oncogenic inputs (strong oncogenic addiction), sufficient to induce cell transformation possibly in association with a limited number of additional synergizing defects. On the contrary, ALK− ALCLs might require the acquisition of a large number of tumorigenic defects, which

could accumulate overtime resulting in a highly heterogeneous karyotype.

Morphologically ALCLs can be divided in five morphologic variants (a common, giant cell-rich, lympho-histiocytic, small-cell type, and Hodgkin-like, with the common variants corresponding to the lesions originally described by Stein et al., 2000). Cytologically, the neoplastic cells show a wide spectrum, although all cases have a variable proportion of "hallmark cells" (Benharroch et al., 1998; Jaffe, 2001; **Figure 1**). These are large lymphocytes with "horse shoe" or "kidney shaped" nuclei and abundant cytoplasm. ALCLs have a unique immunophenotype characterized by the strong expression of the CD30 antigen, a cytokine receptor of the tumor necrosis factor receptor superfamily, with a membranous pattern often associated with reinforcement staining within the Golgi area. The neoplastic cells, which are of T-cell origin, commonly lack many of the T-cell markers, although they frequently express cytotoxic molecules such as granzyme B, perforin, and T-cell-restricted intracellular antigen-1 (TIA1). Moreover, ALK+ ALCLs frequently display phosphorylated peptides including pSHP2 and pSTAT3 (**Table 3**).

ANAPLASTIC LYMPHOMA KINASE

Anaplastic lymphoma kinase is a tyrosine kinase receptor (TKR), with an extracellular ligand-binding domain, a transmembrane-spanning region and an intracellular tyrosine kinase domain. It belongs to the Insulin Receptor superfamily and is closely related to the leukocyte tyrosine kinase (LTK). The overall structure of ALK has been conserved through evolution; this is particularly the case for the kinase domain, which is highly homologous among a variety of species, even though some differences, including the Tyr1604, have been found, suggesting peculiar functions in different species and/or in unique pathological settings (Bai et al., 1998).
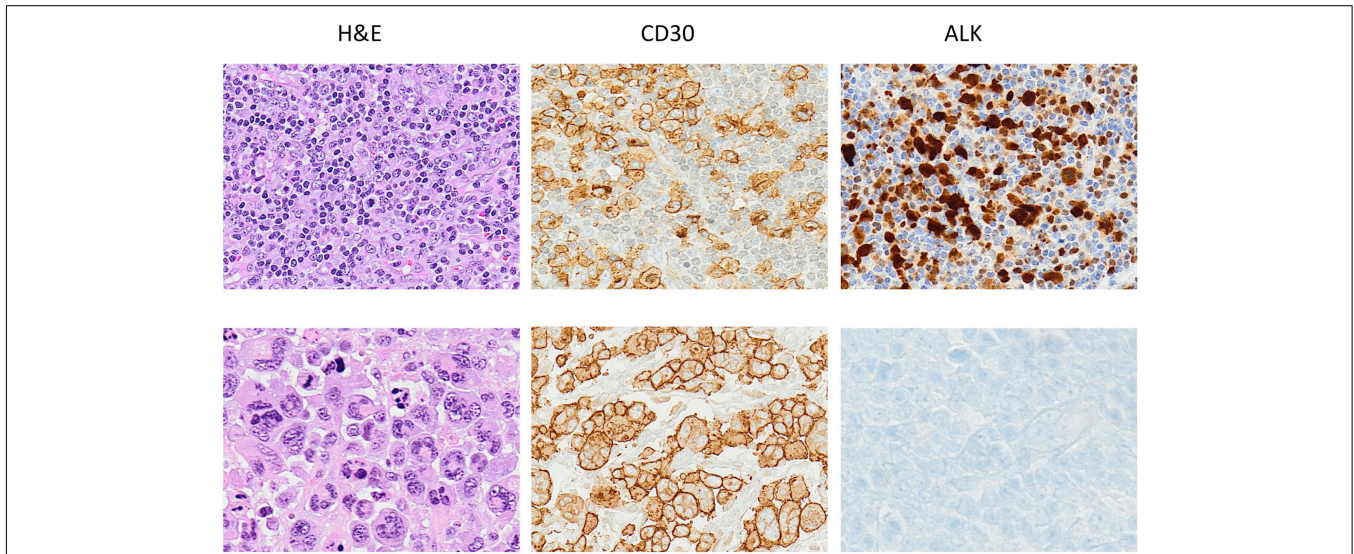
FIGURE 1 | Anaplastic large cell lymphoma. The neoplastic cells of ALCL typically show a broad spectrum of morphologic features, and virtually all cases display a variable proportion of "hallmark cells." The CD30 antigen, a cytokine receptor of the tumor necrosis factor receptor superfamily, is strongly expressed by all ALCL often in association with perforin, granzymes and T1A1 antigens. Alk expression is detected only in a subset of ALCL as result of translocations involving the ALK gene.

Table 3 | Immunophenotypic and genomic features of ALCL.

	ALCL	
	ALCL ALK+	ALCL ALK−
IMMUNOPHENOTYPIC FEATURES		
CD30	+++	+++
CD2	+	++
CD3	−/+	+
CD4	+/++	+/++
CD8	+	+
CD43	+/++	+/++
CD56	−/+	−/+
TIA1, granzyme B, or perforin	+++	+++
NFTAC	−	−
pSHP2	+/++	−/+
p-STAT3	+++	+
C/EPBβ	+++	+
PDGFRα	−/+	−/+
EMA	+++	++
CD15	−/+	−/+
PAX5	−	−
GENETICS		
TCR γ/β	+++	+++
IgH/L	−	−
EBV	−	−

+++, X > 75%; ++, 50% < X < 75%; +, 25% < X < 50%; −/+, X < 25%. −, X = 0%.

The physiological role of ALK has been only partially defined. In *C. elegans* and *Drosophila*, the loss of ALK results in defects in midgut development, neuronal wiring, and plasticity. In adult mice, the genomic deletion of ALK has subtle or no phenotype, although its expression is upregulated after neuronal injuries enhancing the regeneration of myelinated axons (Mi et al., 2007). Recent data have been confirmed that loss of ALK signaling results in a dramatic decrease in newborn neurons in young as well as adult mice, as in *Drosophila* where ALK protects larval neurogenesis from nutrient stress (Cheng et al., 2011). Interestingly, ALK in mammals appears to enhance cognitive performance as well (Weiss et al., 2012).

In ALCLs, the ALK gene is inappropriately expressed in lymphoid cells as a result of several translocations, in which the intracytoplasmic region of the gene, coded by the exons 20–29 (NM_004304.3), is fused with different partners that provide dimerization domains (**Table 4**; Pulford et al., 2001; Chiarle et al., 2008b). The most common of these translocations is the t(2;5; p23;q35) that leads to the nucleophosmin (NPM)–ALK fusion protein (Chiarle et al., 2008b). Alternative translocations of ALCL have been discovered involving various partners, including the TPM (Lamant et al., 1999), TFG (Hernandez et al., 1999, 2002), ATIC (Colleoni et al., 2000), TSPYL2 (Touriol et al., 2000), MSN (Tort et al., 2001), KIAA1618 (Cools et al., 2002), VCL (Debelenko et al., 2003), MYH9 (Lamant et al., 2003), KIFB5 (Wong et al., 2011; Takeuchi et al., 2009). Notably, ALK+ ALCL often display additional alterations involving many chromosomes (deletions:4, 9p, 10p, 11q, and 13q, and gains: 1q, 7p, 6p, 17p, and 17q; Ott et al., 1998; Zettl et al., 2004; Salaverria et al., 2008), although common discrete secondary lesions have not been discovered yet. Moreover, using conventional genomic approaches, and more recent next generation sequencing from fresh and/or paraffin embedded tissue samples have shown new translocation of ALK in many types of human cancers (**Table 4**; Jung et al., 2012; Lipson et al., 2012; Takeuchi et al., 2012; Togashi et al., 2012).

Table 4 | Recurrent chromosomal translocations involving the ALK gene in human cancers.

Disease	Chromosomal abnormalities	Fusion protein (kDa)	Partner	Frequency (%)	ALK IHC staining	Reference
ALCL	t(2;5)(p23;q35)	NPM–ALK (80)	*NPM1*	75–80	Cyto/nuclear	Morris et al. (1994), Shiota et al. (1994)
ALCL	t(2;17)(p23;q25)	ALO17–ALK (ND)	*ALO17*	<1	Cyto	Cools et al. (2002)
ALCL	t(2;3)(p23;q21)	TFG–ALK (113)	*TFG*	2	Cyto	Hernandez et al. (1999, 2002)
ALCL	t(2;X)(p32;q11–12)	MSN–ALK (125)	*MSN*	<1	Cyto	Tort et al. (2001, 2004)
ALCL	t(1;2)(q25;p23)	TPM3–ALK (104)	*TPM3*	12–18	Cyto	Lamant et al. (1999), Siebert et al. (1999)
ALCL	t(2;19)(p23;p13)	TPM4–ALK (95)	*TPM4*	<1	Cyto	Meech et al. (2001)
ALCL	inv(2)(p23;q35)	ATIC–ALK (96)	*ATIC*	2	Cyto	Ma et al. (2000), Trinei et al. (2000), Colleoni et al. (2000)
ALCL	t(2;22)(p23;q11.2)	MYH9–ALK (220)	*MYH9*	<1	Cyto	Lamant et al. (2003)
ALCL	t(2;17)(p23;q23)	CLTC1–ALK (250)	*CLTC1*	2	Cyto	Touriol et al. (2000)
DLBCL	t(2;5)(p23;q35)	NPM–ALK (80)	*NPM1*	N/A	Cyto/nuclear	Adam et al. (2003), Onciu et al. (2003)
DLBCL	t(2;17)(p23;q23)	CLTC1–ALK (250)	*CLTC1*	N/A	Granular cyto	De Paepe et al. (2003)
DLBCL	t(2;5)(p23.1;q35.3)	SQSTM1–ALK (ND)	*SQSTM1*	N/A	Cyto	Takeuchi et al. (2011)
DLBCL	ins(4;2;4)(?;q21) t(2;4)(p24;q21)	SEC31A–ALK (ND)	*SEC31A*	N/A	Cyto	Bedwell et al. (2011), Van Roosbroeck et al. (2010)
IMT	t(1;2)(q25;p23)	TPM3–ALK (104)	*TPM3*	50	Cyto	Lawrence et al. (2000)
IMT	t(2;19)(p23;p13)	TPM4–ALK (95)	*TPM4*	<5	Cyto	Lawrence et al. (2000)
IMT	t(2;17)(p23;q23)	CTLC–ALK (250)	*CLTL*	<5	Cyto	Bridge et al. (2001), Patel et al. (2007)
IMT	inv(2)(p23;q35)	ATIC–ALK (96)	*ATIC*	<5	Cyto	Debiec-Rychter et al. (2003)
IMT	t(2;11;2) (p23;p15;q31)	CARS–ALK (ND)	*CARS*	<5	Cyto	Cools et al. (2002), Debelenko et al. (2003)
IMT	t(2;2)(p23;q13) inv(2)(p23;p15;q31)	RANBP2–ALK (ND)	*RANBP2*	<5	N/M	Ma et al. (2003), Marino-Enriquez et al. (2011)
IMT	t(2;4)(p23;q21)	SEC31L1–ALK (ND)	*SEC31L1*	<5	Cyto	Panagopoulos et al. (2006)
ESCC	t(2;19)(p23;p13)	TPM4–ALK (110)	*TPM4*	N/A	Cyto	Jazii et al. (2006), Du et al. (2007)
RCC	t(2;10)(p23;q22)	VCL–ALK (117)	*VCL*	N/A	Cyto	Debelenko et al. (2011)
NSCLC	inv(2)(p21;p23)	EML4–ALK (120)	*EML4*	2–5	Cyto	Rikova et al. (2007), Soda et al. (2007)
NSCLC	t(2;3)(p23;q21)	TFG–ALK (113)	*TFG*	2	Cyto	Rikova et al. (2007)
NSCLC	t(2;10)(p23;p11)	KIF5B–ALK (ND)	*KIF5B*	<1	Cyto	Takeuchi et al. (2009), Wong et al. (2011)
NSCLC	t(2;14)(p23;q32)	KLC1–ALK (ND)	*KCL1*	<5%	Cyto	Togashi et al. (2012)
NSCLC	t(2;9)(p23;q31)	PTPN3–ALK (ND)	*PTPN3*	N/A	Cyto	Jung et al. (2012)
CRC	inv(2)(p21;p23)	EML4–ALK (120)	*EML4*	<5%	Cyto	Lin et al. (2009)
CRC	t(2;2)(p23.3)	C2orf44–ALK (ND)	*C2orf44*	N/A	Cyto	Lipson et al. (2012)
BC	inv(2)(p21;p23)	EML4–ALK (120)	*EML4*	<5%	Cyto	Lin et al. (2009)

ND, not determined; Cyto, cytoplasmic; N/M, nuclear/membrane; N/A, non-assessed; ALCL, anaplastic large cell lymphoma, DLBCL, diffuse large B-cell lymphoma; IMT, inflammatory myofibroblastic tumor; ESCC, esophageal squamous cell cancer; RCC, renal cell cancer; NSCLC, non-small-cell lung cancer; CRC, colon–rectal cancer; BC, breast cancer.

ALK SIGNALING

Understanding how ALK signals and defining the mechanisms responsible for its deregulation are critical to dissecting how ALK mediates cellular transformation and provides the basis for "intelligent" therapeutic approaches.

An area of debate remains on how the ALK receptor physiologically signals in mammals. Contrary to Drosophila in which the dALK ligands have clearly been identified (Palmer et al., 2009), in mammals several hypothetical ligands have been considered, among them are: PTN (pleiotrophin or HB-GAM), OSF-1(osteoblast-specific factor-1), HARP (heparin affinity regulatory peptide), HBNF (heparin-binding neurotrophic factor), and MK (midkine). Nevertheless the role of these molecules

remains controversial and it is plausible that other, so far unknown ALK ligands, may exist. It can also be contemplated that some of the current ligands, such as PTN, may function indirectly inhibiting alternative molecules like the phosphatase RPTPβ/ζ, providing a higher steady state phosphorylation status of the receptor (Perez-Pinera et al., 2007).

The ectopic expression of ALK chimera leads to their hetero- and homo-dimerization, via the dimerization domains of ALK partners resulting in a constitutive activation of the intracytoplasmic catalytic domain. This results in the constitutive activation of multiple signaling pathways, which lead to cell transformation and sustains the neoplastic phenotype (Chiarle et al., 2008b). In NPM–ALK ALCL, the N-terminus region of NPM1

provides the dimerization domain essential for the chimera's auto-phosphorylation. NPM1 is a multifunctional protein, which acts as a molecular chaperone in the transport of pre-ribosomal particles from the nucleus to the cytoplasm, but it functions in addition to DNA repair, transcription, and genomic stability regulation (Okuwaki, 2008).

It is well established that the forced expression of ALK chimeras has oncogenic potential in *in vivo* as well *in vitro* models (Kuefer et al., 1997; Bai et al., 1998; Chiarle et al., 2005; Turner and Alexander, 2005). *In vivo* the constitutive expression of ALK transgenic cassettes under hematopoietic promoters can drive B- or T-cell transformation, providing a valuable tool for the study of ALCL (Chiarle et al., 2003; Turner and Alexander, 2005) and for the set up of innovative chemotherapeutic modalities (Cheng et al., 2012). Similar findings have been recently obtained expressing EML4–ALK (Soda et al., 2008) or TFG-1–ALK fusion proteins under lung-restricted promoters.

The transforming properties and the signaling elicited by ALK fusion proteins have been extensively studied using a variety of genetic, proteomics, and pharmacological approaches. Overall, as in many other oncogenic kinases, the deregulated expression of ALK leads to the simultaneous activation of many signaling pathways, which have been proven to synergistically provide the required signals for the maintenance of the neoplastic phenotype. Indeed, the ablation of the ALK signaling, at least in lymphoid cells, demonstrates the absolute ALK oncogenic "addiction" of these tumors (Piva et al., 2006; Wan et al., 2006; Christensen et al., 2007; Galkin et al., 2007). It is unclear which further defects may contribute in ALK+ ALCL, although it is proven that the forced expression of ALK chimera in normal cells leads to senescence (Martinelli et al., 2011) and that ALK+ lymphoma in Tg mice require a few months to occur, suggesting that a combination of lesions is acquired. Remarkably, the activation of IGF-IR can contribute to the neoplastic phenotype of ALK+ ALCL cell lines (Shi et al., 2009). More importantly, in the case of ALK+ NSCLC cell lines, ALK addiction has been proven to be variable and the patho-genetic role of other kinase receptors have been suggested in these settings (Koivunen et al., 2008). The determination of the ALK dependency and the contribution of other co-signaling kinase(s) is critical for defining the most effective chemotherapeutic regimen in each patient. Thus in selected settings, it may be necessary not only to dissect the presence of any given oncogenetic defect (i.e., EGRF, k-Ras mutation etc.) but to also quantify the levels of the activation of critical targetable molecules as well.

Mutagenesis and functional studies have identified a plethora of NPM–ALK interacting molecules which ultimately lead to the activation of key pathways including RAS/Erk, PLC-γ, PI3K, and Jak/signal transducers and activators of transcription (STAT) pathways, which in turn control cell proliferation and survival and cytoskeletal rearrangements (**Figure 2**).

Ras/Erk PATHWAY

The increased growth of ALK+ ALCL cells is believed to be largely dependent on the activation of the Ras/Erk pathway (Fujimoto et al., 1996; Pulford et al., 2001). In NPM–ALK several adaptors or scaffolding molecules with Src-homology 2 (SH2) or phos-photyrosine binding (PTB) domains can bind and activate the

Ras/Erk pathway. Although, SHC, IRS1, and Grb2 bind directly to NPM–ALK, neither SHC nor IRS1 are essential for transformation, since loss of their ALK docking sites does not preclude cell transformation (Fujimoto et al., 1996). Grb2 binds to different regions of NPM–ALK, mainly Tyr(152–156), Tyr(567), and a proline-rich region, Pro(415–417), and primarily regulates ALK-mediated phosphorylation of SHP2, which plays a key role in ALCL cell growth (Riera et al., 2010). The concomitant down-regulation of ERK-1 and ERK-2 in ALK+ ALCL using RNAi or specific Mek inhibitors leads to a cell cycle arrest in absence of an increased rate of apoptotic cells (Marzec et al., 2007). Ras activation via MAPK (mitogen-activated protein kinases), and ERK-1 and 2, regulates several AP-1 transcription factors, which are believed to contribute to the ALCL neoplastic phenotype (CD30; Watanabe et al., 2005; Hsu et al., 2006; Staber et al., 2007), regulate cytotoxic molecule expression (Pearson et al., 2011) and they are required for ALK-mediated transformation in mouse models.

PCL-γ PATHWAY

PLC-γ, docking in position Y664 of NPM–ALK, induces the hydrolysis of phosphatidylinositol (PIP2) into inositol triphosphate (IP3) and diacylglycerol (DAG), molecules that can modulate the release of Ca^{2+} from intracellular compartments and activates the serine/threonine protein kinase C (PKC). The pathogenetic role of this pathway has been documented in Ba/F3 cells (a pro-B line that requires IL-3 for survival and growth), which can overcome IL-3 requirements in presence of the ectopic NPM–ALK^wt but not NPM–ALK^Y664F (Bai et al., 1998). Interestingly, the PLC-γ docking site (Y664) is present only in the human ALK but not in the mouse gene, suggesting a unique function in human ALK-mediated transformation.

PI3K PATHWAY

NPM–ALK interact directly and most likely indirectly with PI3K (Bai et al., 2000). The ALK–PI3K interaction (catalytic p110 subunit) leads to the activation of the PKB/AKT and the subsequent downstream signaling events. These play a critical role in ALK-mediated transformation, as originally demonstrated by the dominant negative PKB/Akt approach (Slupianek et al., 2001) and by up-regulation of cyclin D2 and down-regulation of Bim-1 and p27 via hyperphosphorylation of the transcription factor FOXO3a after ALK forced expression (Gu et al., 2004). Finally the PI3K/AKT signaling pathway has been shown recently to control the phosphorylation levels of GSK3β, upregulating the expression of MCL-1 and Cdc25A, which may contribute to the neoplastic phenotype (Mcdonnell et al., 2011).

c-SCR PATHWAY

c-Src, a TKR, plays a relevant role in ALCL cell migration, as well as in cell proliferation and growth. c-Src that is normally maintained in a catalytically inactive conformation can be activated by NPM–ALK and it is required to sustain ALCL cell growth (Cussac et al., 2004). Src-family kinases may also contribute to VAV-1 signaling, leading to a sustained activation state of Cdc42. Cdc42 and p130C as in this context regulates ALCL cell shape and migration (Ambrogio et al., 2008).
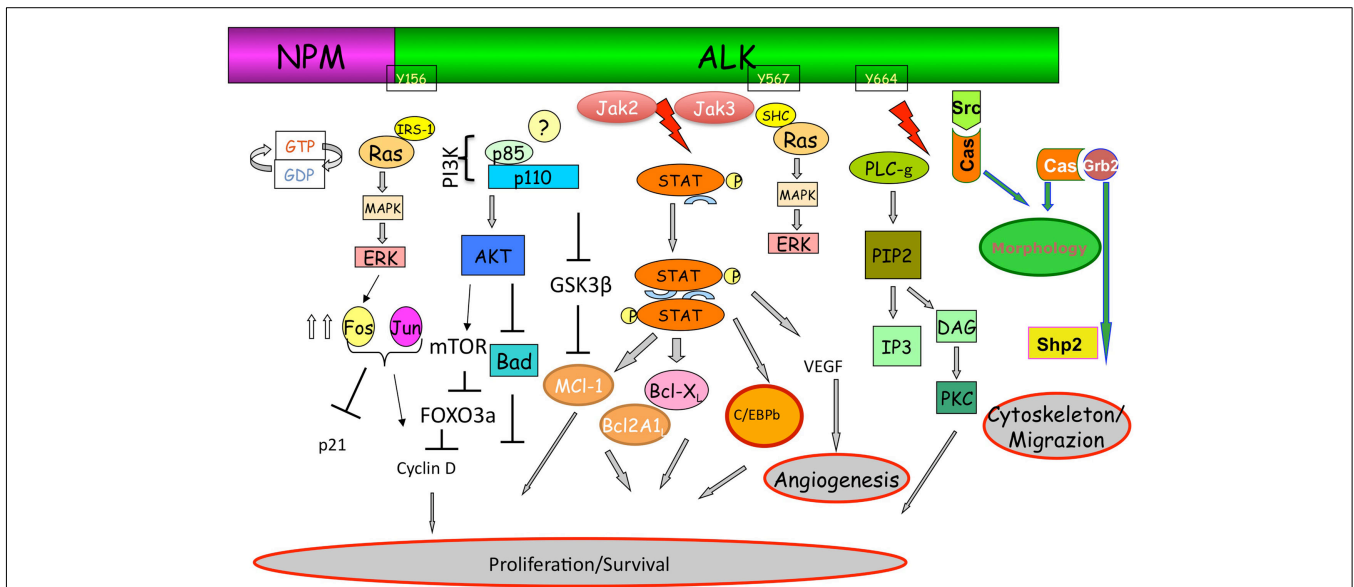
FIGURE 2 | Anaplastic lymphoma kinase signaling. The ectopic expression of ALK fusion proteins leads to the constitutive activation of the tyrosine kinase, and concomitant activation of multiple signaling pathways. ALK, via a direct binding of IRS1, Shc, and Src on specific tyrosine residues, is a strong activator of the Ras/Mek/Erk-1/2 extracellular signal-regulated kinase (Erk) pathway that provides key mitogenic signaling. Ras/Erk pathways, via multiple AP-1 transcription factors, up regulates the CD30 expression. Mitogen-induced extracellular kinase (MEK)/ERK signaling pathway results in phosphorylation of mTOR which in turn leads to the phosphorylation of several critical targets (p70S6K and S6rp, eukaryotic initiation factor 4E (eiF4E)–binding protein-1 (4E-BP1). Activation of mTOR is also achieved through the synergist activation of the phosphatidylinositol 3-kinase (PI3K) pathway as result of the activation of Akt1/2 molecules. In the case of PLC-γ, this protein is directly activated by ALK (Y664) and capable of triggering pro-mitogenic stimuli generating diacylglycerol and IP3, which leads to the activation of PKC, and mobilization of calcium stores from the endoplasmic reticulum as well. As

for many other cells, activated Akt1/2 enhance ALCL cell survival by blocking the function of pro-apoptotic proteins, such as BAD and indirectly regulate key cell cycle regulators (BIM-1, p27, and cyclinD2) via FOXO3A. Negative regulation of p-GSK3β leads to the up-regulation of Mcl-1 and CDC25A which confers the advantage of growth and protection from apoptosis. Stat3 regulated molecules are essential and required for the maintenance of the ALK-mediated neoplastic phenotype of ALCL cells. ALK fusion proteins can directly phosphorylate STAT3, alternatively JAK2/3 can also synergize. Negative regulators of this pathway may impose their action by dephosphorylating ALK itself and/or receptor-associated kinases (Src and JAK). Notably, methylation of the promoter region of SHP1 has been reported in ALK+ ALCL cells, resulting in the down-regulation of its expression and thus an overall enhancement of STAT3 signaling. Activation of STAT3 is associated with a specific signature (∼1500 genes), which includes several transcription factors (i.e., CEBP/β), cell cycle (i.e., Cyclin D, c-myc etc.), survival/apoptosis molecules (Bcl-A2, Bcl-X$_L$, Survivin, MCL-1) and cell adhesion, and mobility proteins.

Jak/STAT PATHWAY

Signal transducers and activators of transcription proteins are a family of transcription factors first characterized for their role in cytokine signaling. These proteins contain a site for specific tyrosine phosphorylation, which after modification results in a conformational rearrangement and dimerization through phosphotyrosine–SH2 domain interactions. Once STATs are phosphorylated, they dimerize and accumulate in the nucleus (Levy and Inghirami, 2006). Alternative non-canonical properties have recently been described for STAT3 (Reich, 2009). The pathogenetic role of both STAT3 and STAT5 have been demonstrated in ALK+ ALCL, although the oncogenic contribution of STAT5 is less clear and most likely linked to the unique experimental models (Nieborowska-Skorska et al., 2001). Indeed, in either T (Zamo et al., 2002; Zhang et al., 2002; Amin et al., 2004) or B (Momose et al., 2009) ALK+ lymphoma p-STAT3 species are most commonly expressed and in ALCL, ALK signaling via STAT3 represses STAT5 expression (Zhang et al., 2007).

The constitutive activation of STAT3 is achieved through multiple mechanisms, such as NPM–ALK which can directly phosphorylate STAT3 as well as JAK3 that can indirectly contribute,

as demonstrated by reduce levels of p-STAT3 after its pharmacological inhibition (Amin et al., 2003). Moreover primary ALCL can display a loss of JAK/STAT pathway negative regulator Shp1 (Honorat et al., 2006) or alternatively demonstrate an overexpression of protein phosphatase 2A, capable of sustaining a STAT3 phosphorylation status (Zhang et al., 2002).

The critical role of STAT3 in ALK+ ALCL models is well established since its knockdown by RNAi invariably leads to first cell cycle arrest (Amin et al., 2004; Piva et al., 2006), followed over time by the execution of irreversible apoptotic program. This phenomenon is associated with well-defined changes in transcription and the reproducible modulation of a large number of genes (∼1500), many of which are physiologically down-regulated in the presence of active p-STAT3. Most of these changes can be observed using anti-ALK inhibitors or anti-ALK RNAi, demonstrating that "oncogenic addiction" of ALK+ ALCL cells requires a STAT3-mediated transcription. Moreover some of these genes can be used as molecular classifiers and may became diagnostic tools for the identification ALCL among primary T-NHL (Chiarle et al., 2008b; Piva et al., 2010). To discover the STAT3 transcriptionally regulated genes, we have recently conducted time kinetics

experiments, demonstrating that only a limited number of known and unknown STAT3 genes are directly modulated in ALCL. These, however, can lead to a cascade of downstream changes. Interestingly enough, this STAT3 dependent phenotype requires the concomitant activation of multiple genes, as demonstrated by the individual knockdown by siRNA and/or the activation of a unique miRNA cluster(s). Intriguingly, in ALK+ NSCLC cell lines the STAT3-associated gene expression signature shares a very limited number of targets with that of ALCL, suggesting that STAT3 oncogenic requirements are lineage restricted and/or cell dependent, as shown by the mild phenotype associated with the knockdown of STAT3 in ALK+ NSLCL cells lines (Takezawa et al., 2011).

STAT3 activation controls cell cycle, apoptosis and DNA damage, adhesion and motility, G protein signaling, inflammation and immune response, metabolic pathways, and angiogenesis. Increased expression of many anti-apoptotic molecules, such as Bcl2, Bcl-XL, Survivin, and Mcl-1 proteins, that in association with ALK-regulated genes such as BCL2A1, provide strong anti-apoptotic signals (Amin et al., 2004; Piva et al., 2006). Uncontrolled proliferation is in part regulated through cyclin D3 (Dalton et al., 2005) and c-myc (Amin et al., 2003). Finally several studies have shown how ALK–STAT3 may control angiogenesis (Marzec et al., 2011) and regulate key immune functions (Marzec et al., 2008). Lastly, STAT3 is in part responsible for the null phenotype of ALK+ ALCL, determining T-cell identity and signaling (Zhang et al., 2002; Ambrogio et al., 2009). The strict requirement for STAT3 activation in ALK+ ALCL makes this molecule an ideal target particularly in the context of mutated ALK chimera, resistant to anti-ALK small molecules. Indeed, small selective compounds like S3I-201, niclosamide, and the pyrimethamine show potent apoptotic effects against ALK+ ALCL cells.

Finally, through proteomic studies many molecules were identified and proven to contribute to the neoplastic phenotype protecting from apoptosis (Bassermann et al., 2005), promoting cell cycle, transport, and transcription activities (Sjostrom et al., 2007). Interestingly, this approach has confirmed the discovery of novel ALK chimera and the definition of a new subset of NSCLC tumors (Rikova et al., 2007). Using quantitative proteomic-based approaches coupled with an enrichment strategy that allows the identification specific phosphorylation sites within peptides, we have shown that 5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase/IMP cyclohydrolase (ATIC) is phosphorylated at Y104 via ALK and that ALK-mediated ATIC phosphorylation enhances its enzymatic activity, dampening the methotrexate-mediated transformylase activity inhibition (Boccalatte et al., 2009).

Many of the information provided above have been derived using ALK+ ALCL cell lines harboring the NPM–ALK chimera. It is postulated that the ALK-mediated transformation of different fusion proteins may be imposed through analogous mechanisms. Indeed, many of the ALK fusions can elicit similar signals. Although, they share dimerization domains, which allow the constitutive activation of the proteins, different partners display unique features and impose specific intracellular localization to the ALK proteins. This implies that each of them may have unique, yet slightly different properties, whose contribution(s) to the neoplastic phenotype remain yet to be clarified. Toward this end, it

has been proven that some fusions display a higher migratory and invasive capacity *in vitro* and *in vivo* (Armstrong et al., 2004). The clarification of these features may be critical in the understanding of the oncogenic potential and oncogenic properties of different ALK fusion proteins in human cancers.

TARGETED THERAPY

The discovery that EML4–ALK fusions can be found in NSCLC has dramatically changed the landscape of ALK, fostering innovative therapies to move very rapidly into the clinical field. Remarkably, the data generated in ALCL and inflammatory myofibroblastic tumors (IMT), although providing the intellectual/scientific bases for the design of drug discovery programs, have not generated sufficient interest from either the pharmaceutical or scientific communities. This perception has definitively changed when (Soda et al., 2007) first described ALK translocations in a set of lung cancers. Since then, we have found that many other human cancers carry ALK lesions in a well-defined subset of patients, making the "ALK story" one of the most attractive and promising of the last decade. Ultimately, the discovery that sporadic as well as familiar neuroblastoma can carry ALK activating mutations has opened additional hopes and new avenues. This has led to the production of different ALK inhibitors that are now in one stage or the other of investigation, with the Crizotinib as the first approved drug for the treatment of lung cancer patients. Although we have witnessed tremendous advances in a very short period of time, many issues remain ahead. These include the design of the most effective chemotherapeutic combinations, the definition of the right combinations for every individual patient, the discovery, early on during treatment, of non-responder patients, and finally new modalities for overpassing the inevitable resistance associated with the usage of anti-ALK inhibitors.

We believe that many of these issues could be solved once a systematic molecular stratification is employed in all cancer patients. The recent application of Next Generation Sequencing in lung and colon cancer has not only showed that novel translocations (Lipson et al., 2012) can be discovered among these tumors but they might provide the basis for identifying the molecular fingerprints responsible for the clinical behavior of individual cancers. This will only be possible when the cost of these analyses are affordable and when the data obtained by friendly bioinformatic modules are immediately translated into daily clinical practice.

These and many other questions have been further discussed in the accompanying manuscripts.

Here, we would like to address specific topics and discuss a few alternatives.

POTENTIAL CHALLENGES

Indeed, we need to carry forward the progress that we have gained in the NSCLC into the field of ALCL, IMT, and neuroblastoma; despite the fact that these diseases represent only a relatively small market and are rare orphan entities. Nevertheless, many of them occur in children and we have very little therapeutic options for some of them. The usage of anti-ALK inhibitors may provide a unique opportunity reaching relevant clinical benefits in the absence of substantial side effects. This is critical in young individuals who may benefit the most. Anti-ALK

inhibitors and innovative approaches are required for refractory or relapsed ALK+ ALCL after conventional treatment. The currently recommended protocol for these patients is an autologous stem cell transplantation (HDC/ASCT) preceded by high-dose chemotherapy in first remission. Alternatively, a few experimental studies currently open are investigating the efficacy of Brentuximab vedotin (SGN-35) alone (NCT01352520, NCT0043084, NCT00866047, etc.) or in combination with conventional drugs (NCT00649584).

There are also clinical studies investigating the usage of Crizotinib (CT00939770) or AP26113 (NCT01449461) ALK inhibitors in these settings.

Finally, since kinase inhibitors are invariably associated to drug resistance and they are incapable of eradicating stem cancer cells, new approaches need to be contemplated. Although ALK signaling is considered to be critical in many ALK+ ALCLs, we have seen that co-synergizing signals may contribute to maintain the neoplastic phenotype (in ALCL IGFR and in NSLCL EGFR, c-Met etc.). These signals often converge on critical downstream molecules, which could be targeted to improve the therapeutic responses. For examples the concomitant treatment with ALK and MEK inhibitors has been shown to increase apoptosis of ALK+ NSCLC cell line *in vitro* providing a rational approach for clinical interventions (Tanizaki et al., 2012). Notably, the application of geldanamycin-based HSP90 inhibitors has also been proven to be efficacious leading to the forced degradation of the wt or mutated ALK fusion proteins (Chen et al., 2010; Katayama et al., 2011). These and other findings (Bonvini et al., 2004) support clinical trials in ALCL patients NCT00117988).

Moreover, since ALK represents a unique onco-antigen, being required for tumor maintenance, expressed in limited tissue compartments and capable of eliciting relevant ALK-specific B and T-cell responses in lymphoma patients (Pulford et al., 2000; Passoni and Gambacorti-Passerini, 2003; Ait-Tahar et al., 2006, 2007; Chiarle et al., 2008a), this provides the basis for the design and implementation of immune-based therapies, which in association with drug-based approaches could lead to the complete control of the disease. Finally, immunological strategies [i.e., anti-CD25 (NCT00801918) anti-CD30 (Ansell et al., 2007; Forero-Torres et al., 2009) and/or CD26 (Ho et al., 2001) antibodies], in combination with conventional or small molecule approaches, are currently considered to enhance anti-tumor responses or to gain the complete eradication of cancer cells.

FUTURE DEVELOPMENTS

Since ALK oncogenic properties rely on the constitutive activation of multiple pathways, it is plausible that combinational therapies may be designed including not only conventional drugs but also small compounds capable of targeting key effectors within ALK signaling pathways (**Figure 2**). Toward this end, the usage of compounds already in clinics for other applications, but recently discovered to have anti-STAT3 activities, are particularly appealing. These include for example niclosamide and pyrimethamine currently used as anthelmintic or anti-parasitic compounds (Takakura et al., 2011), which display promising therapeutic efficacies in ALK+ ALCL models. Analogously, it is also plausible to consider a set of compounds known to down-regulate members of the Ras-Erk pathway. The most promising are represented by the Mek inhibitors (i.e., AZD6244, PD-325901, GDC-0973 etc.), which can lead to ALCL cell cycle arrest and in combination with anthracycline can become powerful treatment modalities. The inhibitor of PI3K and mTOR (Turturro et al., 2002; Marzec et al., 2007; Staber et al., 2007) have also been proposed and in some cases anti-ALK, HSP90 or mTOR inhibitors have proven to be quite efficacious (Chen et al., 2010; Lovly et al., 2011). Finally, since ALK+ ALCL display a strong expression of BCL2A-1[38], it is reasonable to propose the usage of BH3 mimetic drug as single or in combination with conventional compounds as previously reported for the treatment of B-cell NHL (Paoluzzi et al., 2008a,b; Stolz et al., 2008).

Thus, the armamentarium that we have in our end has become very extensive requiring a systematic analysis of all possible combinations. This implies the set up of novel *in vitro* as well *in vivo* models, which faithfully recapitulate the human clinical scenario. More importantly we need to develop a battery of biomarkers capable of stratifying patients and predicting their clinical outcome. Toward this end, the usage of humanized mouse models in which tumor as well human host cells are reconstituting the host represent the new frontier for drug discovery and personalized oncology modalities (Shultz et al., 2007).

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