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# The role of AP-1 and epigenetics in ALCL

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## 1. ABSTRACT

Anaplastic large cell lymphoma (ALCL) is an aggressive, highly proliferative, T-cell lymphoma with increasing incidence worldwide. Anaplastic Lymphoma Kinase (ALK) fusions occur in about 50% of all cases. Most ALK positive cases of ALCL harbor the t(2;5) translocation that leads to expression of Nucleophosmin-Anaplastic Lymphoma Kinase (NPM-ALK). NPM-ALK induces a variety of oncogenic signaling pathways that lead to malignant transformation of T-cells via Activator Protein-1 (AP-1), STAT3 and other (transcription) factors. In addition to the commonly known AP-1 activators Mitogen-Activated Protein Kinases (MAPKs), there are other signaling pathways, such as PI3K/mTOR/AKT, which are implicated in AP-1 activation/expression in ALCL. The AP-1 factor JUNB was shown to drive ALCL proliferation and the expression of the characteristic ALCL Ki-1 antigen, CD30. cJUN and JUNB target *PDGFRB*, thereby leading to tumor progression and dissemination. Furthermore, aberrant gene expression in ALCL is frequently accompanied by changes in epigenetic regulatory mechanisms, such as DNA methylation patterns. Here, we discuss the role of AP-1 in the pathogenesis of ALCL and provide an overview of pathological epigenetic changes in ALCL cells.

## 2. INTRODUCTION

Anaplastic Large Cell Lymphoma (ALCL) was first recognized as a distinct lymphoma category in 1985. ALCL represents a group of highly malignant peripheral T-cell lymphoma, characterized by constitutive proliferation of large CD30 positive blasts with pleomorphic, often horseshoe-shaped nuclei (1-3). The lymphoid origin of ALCL cells was determined from the presence of clonal T-cell receptor (TCR) gene rearrangements and the expression of T-cell lineage-associated antigens. Primary systemic ALCL has a peak incidence in childhood, accounting for approximately 40% of all childhood lymphoma cases, but less than 5% in adults. During disease progression ALCL frequently involves extranodal sites (4, 5). In the fourth edition of the WHO Classification of Tumors of Haematopoietic and Lymphoid Tissues ALCLs are divided into ALK positive, and ALK negative ALCL. However, another category has recently been reported, which is associated with the presence of breast implants, leading to ALCL (iALCL) indicating previously unknown risk factors (6).

ALK is a receptor protein tyrosine kinase with a putative transmembrane domain and an extracellular domain. In healthy individuals its expression is restricted to neonatal brain tissue (7). Most ALK positive ALCLs carry a balanced reciprocal translocation t(2;5) (p23;q35) resulting in a fusion of the *ALK* gene region coding for entire C-terminal-, catalytic-, cytoplasmic-, kinase- domain, with the oligomerization- domain of the nucleophosmin gene (*NPM1*) including its promoter.

Besides *NPM1*, other genes like *TFG*, *TPM3*, *ATIC*, *CLTCL*, *RANBP2* and *MSN* have been identified as *ALK* fusion partners in ALK positive ALCL (8). In all of these cases the result is constitutive expression of activated ALK fusion tyrosine kinase (FTK). NPM-ALK is by far the most extensively characterized ALK FTK. It exhibits its oncogenic potential through proliferative and survival signaling pathways. Important examples are the phosphatidylinositol 3-kinase/AKT pathway (9), signal transducer and activator of transcription 3 (10) and 5 activation (11), Src tyrosine kinase signaling (12) and diacylglycerol kinase (13) as well as PLCγ (14) signaling. These pathways are either upstream of, or interconnected to activator protein 1 (AP-1) activation (15-19).

AP-1 was identified as a transcription factor (TF) already in 1987 (20). It is a sequence-specific DNA binding factor forming a dimeric complex and it comprises various members of the JUN (cJUN, JUNB, JUND), FOS (cFOS, FRA1, FRA2), ATF (activation transcription factor) and MAF (musculoaponeurotic fibrosarcoma) protein families (15). JUN-JUN and JUN-FOS dimers bind to phorbol 12-O-tetradecanoate-13-acetate (TPA) response elements (TRE) within promoter elements (18). AP-1 activity is regulated in a cell type- and state- specific manner by interactions between AP-1 and other TFs, upstream activators and cofactors (15). All AP-1 proteins have a conserved bZIP region, which comprises a basic DNA binding domain and a leucine zipper region. The latter is responsible for the dimerization, thus enabling DNA binding, and accounts for specificity and stability of homo- and heterodimers formed by the various JUN, ATF, and FOS proteins (15, 21). AP-1 regulates the expression of proteins involved in cell differentiation, proliferation and survival (22). Whereas cJUN is a known driver of malignant transformation (23), JUNB acts pleiotropic in tumorigenesis with reported pro- and anti- apoptotic functions (24). AP-1 proteins like cJUN can activate their own expression (15, 21) and therefore can drive a positive self-regulatory loop.

## 3. AP-1 IS IMPLICATED IN THE PATHOGENESIS OF ALCL

The importance of AP-1 proteins in the pathogenesis of Hodgkin lymphomas and ALCL was reported for the first time by Mathas *et al*. in 2002. They reported strong cJUN and JUNB expression in CD30 positive Hodgkin/Reed Sternberg cells (HRS) and ALCL cell- lines but not in other lymphoma entities. Interestingly, in their hands MAPK-independent AP-1 activation promoted tumor cell proliferation. In HRS cells this is conferred via expression of cell cycle regulator CCND2 (cyclinD2), the proto-oncogene *cMET* and the lymphocyte homing receptor CCR7. In the NPM-ALK ALCL cell line Karpas-299 AP-1 activation prevents apoptosis (25). Srzemska *et al.,* described high cJUN, JUNB, cFOS and FRA1 levels in ALCL patient samples in 2003 (26). cJUN may contribute to uncontrolled cell-division and oncogenesis because siRNA mediated *cJUN* silencing inhibits ALCL cell proliferation and abrogates cell cycle progression (27). *JUNB* on the other hand is amplified in primary cutaneous T-cell lymphomas (28). Interestingly, NPM-ALK is capable of activating the mitogenic ERK MAP-kinase, which upregulates *JUNB* via ETS1 (29). JUNB interacts with the *TNFRSF8* (*CD30*) gene promoter and activates *CD30*. Silencing of *JUNB* results in reduced cell growth and colony formation (30). Moreover, in ALCL *JUNB* knockdown decreases AP-1 activity in general and leads to reduced cell proliferation (17). These data suggest an oncogenic role for cJUN and JUNB in the pathogenesis in ALCL.

## 4. AP-1 COMPLEX AND NPM-ALK: NPM-ALK MEDIATES TRANSCRIPTION/ACTIVATION OF AP-1 COMPLEXES

NPM-ALK mimics activated TCR signaling by inducing AP-1 transcription factors, which bind to promoter elements found in a broad array of cytokine genes. AP-1 activation is achieved via classical TCR activation cascade proteins, IRS-1, SHC, PLCγ and subsequent MAPK cascade activation via RAS and ERK (31). Nevertheless, NPM-ALK positive as well as NPM-ALK negative ALCL equally express AP-1 proteins (17). However, Staber *et al*., found in 2007 that target genes of AP-1 transcription factors like *GM-CSFa*, *GM-CSFb*, *ARF-5*, *FAS*, *FASL* and *BCL-3* are stronger expressed in NPM-ALK positive than in NPM-ALK negative ALCL cell lines. This finding goes hand in hand with higher AP-1 activity in NPM-ALK positive compared to NPM-ALK negative ALCL cell lines. AP-1 expression pattern however, was dependent on the cellular origin, and results slightly differed from study to study. In all cases known to the authors of this current article though, JUNB, cJUN, cFOS and FRA proteins seem to be the most relevant AP-1 factors in ALCL (17, 25). Leventaki *et al*., revealed a NPM-ALK induced cJUN activation mediated via the JNK pathway. Transfection assays in HEK293T and Jurkat cells with active and kinase-dead *NPM-ALK* carrying vectors and knock down experiments with *JNK1* and *JNK2* siRNA in NPM-ALK cell lines revealed NPM-ALK dependent phosphorylation and activation of JNKs and cJUN. NPM-ALK dependency of AP-1 transcriptional activity was further shown when cells were treated with the inhibitor WHI-P154, which inhibits ALK enzymatic activity (27, 32).

## 5. AP-1 PROTEIN JUNB TRANSCRIPTIONALLY REGULATES CD30

ALCL and HRS cells exhibit a constant and characteristic cell surface expression of CD30 in all neoplastic cells. CD30 is a member of the tumor necrosis factor receptor (TNFR) superfamily. The *CD30* promoter consists of a downstream promoter element at positions +24 to +39 responsible for transcription start

site selection and a constitutive core promoter region at -38 to -241 containing three SP-1 and two ETS binding sites. Microsatellite sequences between -1,2 kb and -336 bp contain CCAT repeats, which repress the core *CD30* promoter activity (33, 34). In classical Hodgkin's lymphoma CD30 mediates proliferation through the NF-κB pathway via binding to TNFR-associated factors (TRAF) family members in a ligand independent manner (35). In NPM-ALK positive ALCL the recruitment and aggregation of TRAF proteins can be hampered by NPM-ALK, thus abrogating CD30-driven NF-κB signaling (36). The signaling of CD30 in ALCL is therefore less clear and most likely independent from NF-κB. Watanabe *et al.* found CD30 signaling to be mediated by interaction with JUNB through an autoregulatory mechanism. *CD30* promoter activity was controlled by a self-activated CD30-ERK1/2-JUNB signaling loop in ALCL cell lines. Constitutive CD30 expression led to activation of the ERK1/2 MAPK pathway inducing JUNB, which in turn activated the *CD30* promoter through binding an AP-1 site in a microsatellite region, thus relieving the suppression of the *CD30* core promoter in ALCL cell lines (37). Hsu *et al.* also described a functional correlation between NPM-ALK and CD30 protein and reported that NPM-ALK induced CD30 expression. *CD30* itself was regulated at transcriptional level and was mediated by JUNB (38). JUNB expression was in turn regulated by NPM-ALK and was inducible in normal ALK negative HEK293T cells by forced expression of ectopic NPM-ALK. Transactivation of *CD30* via enhanced *CD30* promoter activity mediated by JUNB binding to the AP-1 site occurred in a cell specific manner and was observed in Karpas-299 cells but not in HEK293T (38). Finally, JUNB was shown to be expressed not only in cases of classical Hodgkin lymphoma, cutaneous ALCL and CD30+ diffuse large B-cell lymphoma, but also in lymphomatoid papulosis. Patients with nodular lymphocyte-predominant Hodgkin lymphomas and diffuse large B-cell lymphomas that were CD30 negative did not express JUNB (39).

## 6. AP-1 AND THE ERK1/2, JNK, MTOR AND NF-KB PATHWAY

In many tumor types, increased AP-1 expression is accompanied by activated ERKs (15). Whereas Mathas *et al*. found a MAPK-independent AP-1 activation by NF-κB in HRS cells (25), Staber *et al.* observed a MEK/ERK- JUNB signaling in ALCL cells. *JUNB* transcription was dependent on the activation of the MEK/ERK pathway, and treatment of ALCL cell lines with the MEK-inhibitor UO126 resulted in decreased *JUNB* (mRNA) levels (17). cJUN phosphorylation was not affected following MEK inhibitor (U0126) treatment of ALCL cells, indicating signaling via JNK (27). On a translational level, JUNB was associated with the mammalian target of rapamycin (mTOR) pathway as JUNB protein levels decreased after rapamycin treatment of ALCL cell lines. Consequently, *JUNB* mRNA shifted from larger polysomes into monosomes and RNPs. Translational regulation of JUNB was presumed since *JUNB* mRNA harbors a 5'TOP-like motif in its 5'-UTR near the transcriptional start site (16). Pharmacological inhibition of mTOR could therefore represent an alternative in the treatment of ALCL.

## 7. IMMUNMODULATORY EFFECTS OF AP-1

NPM-ALK positive ALCLs are of T/null immunophenotype and the normal cellular counterpart of an ALCL cell is presumed to be the cytotoxic T-lymphocyte. Cytotoxic T-cells as well as ALCL tumor cells often bear cytotoxic granules like Granzyme B (GzB) or Perforin in the cytoplasma. Interestingly, JUNB binds to the *GzB* promoter and acts as a direct transcriptional *GzB* activator. Moreover, *GzB* as well as *perforin* transcription are promoted by NPM-ALK. Signaling through NPM-ALK and JUNB affects the expression of cytotoxic molecules in NPM-ALK positive ALCL. Therefore, Pearson, Lee *et al*., came to the interesting conclusion that in NPM-ALK positive ALCL - GzB and perforin are not only expressed due to the T-cell origin but also are actively promoted by oncogenic signaling (40). Interestingly, AP-1 might also be involved in immune system modulation by ALCL tumors. Galectin-1 (GAL1) is an immune- modulatory glycan- binding- protein regulated by an AP-1-dependent promoter element that leads to an immune-suppressive T-cell environment. Together with AP-1, GAL1 was selectively expressed in malignant HRS- as well as in ALCL- cells. Therefore, Rodig *et al*., suggest a common mechanism for tumor immunotolerance in these diseases (41).

## 8. AP-1 AND PDGFR-B

Laimer *et al*, used a *CD4*-NPM-ALK mouse model, with endogenous T-cell lymphoma development at about 8 weeks after birth, to study the role of AP-1 in lymphoma development. They reported a prolonged overall survival, reduced tumor cell proliferation and diminished spread of tumor cells when both AP-1 genes *cJUN* and *JUNB* were conditionally deleted in T cells of NPM-ALK transgenic mice. Deletion of either one of the two AP-1 components showed no effect on survival rates. Further, the authors deduced that *PDGFRB,* which carries an AP-1 consensus sequence within its promoter, is a direct transcriptional target of cJUN and JUNB, and that PDGFRB is a major factor in driving proliferation, tumor cell survival and tumor dissemination in NPM-ALK positive ALCL. Most interestingly, the tyrosine kinase inhibitor imatinib, which can also target PDGFRB, substantially increased overall survival of CD4-NPM-ALK mice resulting in reduced tumor cell proliferation and enhanced apoptosis. The observed tumor size reduction directly correlated with reduced PDGFRB expression. Also, PDGFRB and PDGFRA expression was observed in a high proportion of human ALK- positive and ALK- negative ALCL samples (42-44)

However, PDGFRB expressing ALK- positive tumors have a significantly worse prognosis than ALCL which do not express the PDGFRB (Kenner *et al*., unpublished). In one ALCL patient so far, off label use of imatinib resulted in complete and sustained clinical remission after 10 days of imatinib treatment (43). In addition, imatinib treatment was extended to other T-cell lymphomas sometimes with surprising success (45). Recently lobatin B treatment was shown to inhibit the expression of NPM/ALK, JUNB and PDGFRB. This was due to cell cycle arrest of ALCL cells in late M phase, resulting in attenuated proliferation rates (42). It might also be worthwhile to elucidate the function of PDGFRs in NPM-ALK negative ALCLs, which has not been done so far.

## 9. CELL CYCLE

cJUN and JUNB can promote cell-cycle progression through regulation of cell cycle checkpoints. Using NPM-ALK positive ALCL cells, Leventaki *et al*., reported in 2007 that inhibition of JNK activity reduces cell proliferation due to G2/M cell-cycle arrest. Furthermore, siRNA mediated silencing of cJUN led to a decreased S-phase cell-cycle fraction and upregulation of p21 and downregulation of CCND3 (CyclinD3) and CCNA2 (CyclinA2) (27).

A comparable however, different effect was achieved by transient knockdown of JUNB with si-RNA in NPM-ALK positive cell lines by Staber *et al.* Cellular proliferation was decreased as evidenced by proliferation curves as well as reduced cell count in the G2/M phase of the cell cycle (17). The effects of JUNB are highly dependent on the cellular context though. While JUNB inhibits the cell cycle by $p16^{INK4a}$ induction and CCND1 (CyclinD1) repression in many cell types (15), it was also reported to promote proliferation by *CCNA2* transcription. Mechanistically, RNAi mediated knockdown of *JUNB* led to downregulation of CCNA2,, CCND2 (CyclinD2) and CCND3 (CyclinD3) but activation of cyclin-dependent kinase inhibitors CDKN2A(p14) and CDKN1A(p21), resulting in cell cycle arrest. In addition, silencing of JUNB sensitized ALCL cells to standard chemotherapeutic agents (30). Another interesting notion on enhanced JUNB expression in ALCL and its consequences was recently provided by Pérez-Benavente *et al.* (46, 47). The authors showed that JUNB undergoes phosphorylation-dependent ubiquitylation during the G2 phase of the cell cycle, which results in its degradation. GSK3 was identified as the kinase and SCF (FBXW7) as the E3 ubiquitin ligase responsible for JUNB degradation in G2. JUNB proteolysis in G2 therefore, seems to be an essential step for proper mitosis. Constitutively activated ALK inhibits GSK3β activity in ALCL cells, which abrogates JUNB degradation and causes CCNA2 up-regulation in mitosis. This can result in genetic aberrations typical for cancer (15, 46, 47).

## 10. MICRO RNA-155 INFLUENCES AP-1

Micro RNA (miR)-155 expression alters many signaling pathways including most prominently the MAPK cascade. MiR-155 induces 3'UTR shortening and isoform switching of MAPK related genes. In addition, it correlates with protein phosphorylation of MAPK components and MAPK downstream targets ERK1/2 and AP-1 members FRA1 and cFOS. MiR-155 was also reported to induce expression of MAPK regulated genes *Zeb1*, *Snail*, *Plaur*, and *SerpinE1* in 2014 (48). Interestingly, Merkel *et al*., identified a set of miRNAs that are specifically deregulated in ALCL in 2010 (49). Amongst other findings they reported a four miRNA classifier that distinguishes NPM-ALK positive from NPM-ALK negative ALCL. The miR-17–92 cluster was stronger expressed in NPM-ALK positive ALCL while miR-155 was expressed at about 10-fold higher levels in NPM-ALK negative ALCL (49). It is suggested that miR-155 can act as a tumor driver in NPM-ALK negative ALCL and other mature T-cell lymphomas and should be considered as a therapeutic target for this class of diseases (Merkel *et al*. 2015, in press).

## 11. EPIGENETIC DEREGULATIONS IN ALCL

Besides genetic mutations, epigenetic alterations are frequently found in diverse malignant diseases (50). Among these alterations, DNA methylation is an epigenetic modification occurring at CpG sites in the genome that normally leads to silencing of the underlying DNA sequence. Cancer cells show a global loss of DNA methylation, which adds to genomic instability and de-regulation of tissue-specific or imprinted genes. Additionally, local hypermethylation can arise at formerly unmethylated promoter CpG islands, often affecting tumor suppressor genes that control cell cycle, apoptosis or DNA repair (51, 52).

So far, a couple of genes important for cell proliferation and survival have been shown to be silenced by promoter methylation in NPM-ALK lymphomas, for example the cell cycle inhibitor $p16^{INK4a}$, the cytokine TNF-, which can trigger apoptosis, and nuclear factor of activated T cells 1 (NFATC1), which is able to transduce pro-apoptotic signals (53-55). Another pro-apoptotic member that is epigenetically silenced in NPM-ALK cell lines and lymph node biopsies from patients is BIM (56). BIM silencing occurred through recruitment of the methyl-binding protein MeCP2 and the SIN3a/histone deacetylase 1/2 corepressor complex, which could be reverted by the histone deacetylase inhibitor trichostatin A or demethylating drugs. In other mechanistic models, the NPM-ALK dependent transcription factor STAT3 has been implicated to contribute to epigenetic silencing in NPM-ALK cells by up-regulating and recruiting DNMT1 to the promoter of certain tumor suppressors such as SHP1 and STAT5A, which stimulate degradation or inhibit expression of NPM-ALK, respectively (57, 58). The IL-2R common

gamma chain which is important for the maturation of normal CD4+ T lymphocytes, is also epigenetically silenced in a STAT3-dependent manner in NPM-ALK cells (59). Besides association with DNMT1, STAT3 might act by suppressing the expression of miR-21, which inhibits DNMT1 mRNA expression. Furthermore, components of the TCR pathway, such as ZAP70, CD3 and SLP76 are silenced by promoter methylation in the disease (60), Genome-wide DNA methylation analyses in our own lab indicate that several additional components of the TCR pathway are silenced by promoter DNA methylation in primary ALK+ ALCLs (Hassler *et al*., unpublished results). Data is accumulating showing that epigenetic silencing of tumor suppressor genes plays a key role in malignant transformation by NPM-ALK. Thus, reversal of these modifications by inhibiting enzymes involved in establishing DNA methylation patterns could be a promising strategy to target aberrant DNA methylation in NPM-ALK positive ALCL (61). The most successful drugs targeting DNA methylation enzymes are the nucleoside analogues 5-azacytdine (5-aza-CR, Vidaza®) and its more stable deoxy-derivative 5-aza-2'-deoxycytidine (5-aza-CdR, Dacogen®) (51). The drugs work via incorporation into DNA of actively proliferating cells. Upon incorporation, they form covalent complexes with DNA methyltransferases and thus trap the enzymes at DNA sites. Thereby, they inhibit propagation of DNA methylation during each round of replication at low doses, whereas at high doses cytotoxic side effects can occur (62-64). Indeed, 5-aza-CdR has been shown to be effective in inhibiting proliferation and inducing apoptosis in NPM-ALK cell lines *in vitro* and in xenografts (65). Furthermore, 5-Aza-CdR was able to re-activate expression of tumor suppressors specifically silenced in NPM-ALK such as $p16^{INK4A}$, STAT5A or BIM (56, 58, 65) thus making reversal of DNA methylation by DNMT inhibitors a promising alternative therapy option for NPM-ALK positive lymphomas.

A current issue in NPM-ALK related research relates to the identification and exact characterization of the cell-of-origin of NPM-ALK positive ALCL. Gene expression studies have shown that NPM-ALK positive lymphomas most closely resemble activated CD3+ T-cells, but recent experiments have discovered that a side population resembling early thymic progenitor cells is able to give rise to the bulk tumor in NPM-ALK positive ALCL (66, 67). DNA methylation patterns are characteristic for each cell type from precursor cells to committed or terminally differentiated lineages. Thus, genome-wide analyses of DNA methylation of NPM-ALK tumor cells compared to different stages of T cell development including lymphoid progenitors and T-cell subsets might provide an elegant means to track down the cell-of-origin in NPM-ALK positive ALCL (68, 69).

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