

Short Research Communication

Anti-ALK Antibodies in Patients with ALK-Positive Malignancies Not Expressing NPM-ALK

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Abstract

Patients with Nucleophosmin (NPM)- Anaplastic Lymphoma Kinase (ALK) fusion positive Anaplastic Large Cell Lymphoma produce autoantibodies against ALK indicative of an immune response against epitopes of the chimeric fusion protein. We asked whether ALK-expression in other malignancies induces specific antibodies. Antibodies against ALK were detected in sera of one of 50 analysed ALK-expressing neuroblastoma patients, 13 of 21 ALK positive non-small cell lung carcinoma (NSCLC) patients, 13 of 22 ALK translocation-positive, but NPM-ALK-negative lymphoma patients and one of one ALK-positive rhabdomyosarcoma patient, but not in 20 healthy adults. These data suggest that boosting a pre-existent anti-ALK immune response may be more feasible for patients with ALK-positive NSCLC, lymphomas and rhabdomyosarcomas than for tumours expressing wild-type ALK.

Key words: ALK-antibody titre, NSCLC, neuroblastoma, lymphoma

Introduction

The expression of the anaplastic lymphoma kinase (ALK) is silenced after birth with exception of a few scattered neurons in the central nervous system¹. Almost 90% of Anaplastic Large Cell Lymphoma (ALCL) in children and adolescents carry a reciprocal translocation t(2;5)(p23;q35) fusing the *Anaplastic Lymphoma Kinase (ALK)* gene to *Nucleophosmin 1 (NPM1)*²⁻⁴. The resulting NPM-ALK fusion protein is an almost ideal oncoantigen due to its limited distribution in normal tissues and key roles in

oncogenic processes¹. An adaptive immune response to the resulting oncogenic fusion protein has been described in patients with NPM-ALK-positive ALCL⁵⁻⁸. Almost all the patients generated autoantibodies specific for the oncoantigen, whereas antibodies could not be detected in healthy adults or children or adults with tumours not expressing ALK^{6,8,9}. The magnitude of the antibody response inversely correlates with the risk of relapse^{8,10}. Hence, antibody titres might serve as a surrogate for the

existence and strength of an adaptive B- and/or T-cell response against the aberrant expression of the NPM-ALK protein in ALCL, thereby linking the immune response against NPM-ALK with the ultimate control of NPM-ALK-positive ALCL. As oncogenic rearrangements of ALK or full-length ALK expression have now been described in other cancers, ALK might, serve as an embryonal tumour-associated antigen in all ALK-positive malignancies. Indeed, ALK vaccination has been shown to be effective in preventing disease relapse in a murine tumour model of ALCL and non-small cell lung cancer^{7,11}.

The existence of an anti-ALK autoantibody response in other cancers associated with aberrant ALK expression might also implicate the use of ALK-directed immunotherapeutic strategies for these diseases. For example, about 10% of childhood ALK-positive ALCL and the majority of ALK-positive Diffuse Large B Cell Lymphomas (DLBCL) express ALK-fusion proteins with variant fusion partners (X-ALK) besides NPM1^{2,3,12}. As the contribution of the NPM1 portion of the fusion protein to the induction of the immune response is not known, it remains to be investigated whether X-ALK fusion proteins likewise initiate an immune response.

Aberrant ALK expression also characterises other malignancies. ALK rearrangements occurs in 3-7% of Non-Small Cell Lung Cancer (NSCLC) patients, where an intrachromosomal inversion involving the *ALK* gene locus (*inv(2)(p21p23)*) and the *echinoderm microtubule associated protein 4 (EML4)* gene represents the most common rearrangement detected¹³. ALK positive NSCLC is associated with a younger age at diagnosis, a non-smoking history and the absence of *EGFR* mutations^{14,15}. Cellular expression of EML4-ALK protein is lower than that usually observed in ALK-expressing ALCL rendering it difficult to detect by immunohistochemistry (IHC) with the ALK1 monoclonal antibody¹⁵. Overexpression of full length *ALK* by amplification has been described in 80% of alveolar rhabdomyosarcomas (RMS) and 20% of embryonal RMS. ALK expression is driven by amplification of the *ALK* gene and by increased transcription induced by the tumour-specific PAX3-FOXO1 transcription factor binding to the third intron of the gene^{16,17}. The majority of neuroblastomas, the most common extracranial solid tumour in children, express full length *ALK*^{18,19}. The strength of ALK-expression differs and is influenced by *ALK*-amplifications or modified by *ALK*-mutations²⁰.

In order to investigate whether ALK-expression in human tumours generally induces an immune response, we analysed the presence of circulating ALK-specific antibodies in patients with

ALK-positive, NPM-ALK negative ALCL, DLBCL, NSCLC, neuroblastoma and RMS as well as further 20 healthy volunteers.

Patients and Methods

Study population

Lymphoma cases were selected from patients registered on the ethically approved trials NHL-BFM95 or ALCL99 in Germany between 1995 and 2010 with a diagnosis of ALK-positive ALCL or ALK-positive DLBCL by national reference histology. The patients/parents had given their informed consent to use the material. Selection of cases was based on the presence of ALK-staining restricted to the cytoplasm, the molecularly confirmed absence of *NPM-ALK* fusion and the availability of serum or plasma samples taken at diagnosis. The DLBCL cases were shown to express Clathrin (CLTC)-ALK by Clathrin-ALK specific RT-PCR². Comparison of the immunohistochemical ALK staining pattern with molecular analyses revealed complete concordance with our previous study²; NPM-ALK positive ALCL expressed ALK in the nucleus as well as the cytoplasm while variant ALK fusion proteins lacked nuclear ALK-staining.

Neuroblastoma patients were selected from a German cohort enrolled onto and treated according to the ethically approved NB97 and NB 2004 trials with informed consent. Patients with ALK-expressing tumours detected by real-time PCR and available serum samples during the first four weeks after initial tumour biopsy were included in this study. Mutations were analysed by sequencing.

NSCLC patients (n=21; 7 female, 14 male; age range 33-83, median age 59; 14 never smokers) were treated at the Colorado Cancer Center. All NSCLC patients had stage IV disease at the time of blood sampling and were proven ALK-rearrangement positive by FISH as previously described²¹. Two patients were on no treatment at the time of sampling, all others were receiving treatment with either a licensed or experimental ALK inhibitor (Crizotinib, n=12; LDK378, n=4; AP26113, n=2) or pemetrexed (n=1).

The patient with ALK-positive alveolar rhabdomyosarcoma stage IV showed ALK-staining by IHC and was included after informed consent for measuring ALK antibodies had been secured.

20 healthy adults were included as control group in a study about the characterisation of the cellular and humoral ALK-immune response approved by the ethical committee of the corresponding authors (number: 193/11) after written informed consent.

Detection of the antibody response against ALK

Cyto-centrifuge preparations of COS-1 cells (DSMZ, Braunschweig, Germany) transiently transfected with *pcDNA3* expression plasmids harbouring the entire coding sequences of *NPM-ALK*, *Tropomyosin 3 (TPM3)-ALK*, *EML4-ALK*, full length *ALK* or *pcDNA3* vector only were prepared and incubated with patient's serum diluted 1/50 and 1/100. Patients with a positive staining result were further analysed using serial 3 fold dilution of the serum from 1/250 to 1/60750 as previously described⁸. Antigen-antibody complexes were visualised by indirect immunoperoxidase staining using HRP-rabbit anti-human IgG antibody and diaminobezidine-tetrahydrochloride. The cut-off for a positive result was taken as the highest dilution before the staining of the ALK transfectants was no longer visible by two independent observers⁵. Cos-1 cells transfected with *pcDNA3* vector only were used to control for background as well as false positive staining.

Results and Discussion

We studied 94 patients with ALK-positive, NPM-ALK negative malignancies and 20 healthy control persons for the presence of anti-ALK titres as a surrogate for the existence and strength of the immune response against ALK. The range of malignancies examined included Lymphoma (ALCL and DLBCL), neuroblastoma, NSCLC and one case of rhabdomyosarcoma.

ALK-positive lymphoma was diagnosed in 22 children/adolescents at a median age of 12.4 years (range 2.3-18.2 years). All tumours had ALK translocations but were *NPM-ALK* fusion negative. Of the 20 patients diagnosed with X-ALK positive ALCL 12 (60%) mounted a specific antibody response against ALK with titres ranging from 1/250 to 1/60750 (median 1/750; Table 1). This is a lower incidence than previously reported for patients with NPM-ALK-positive ALCL of whom 90% produce ALK-antibodies although it must be considered that the numbers of patients assessed in this study is considerably lower^{8,9}. ALK-specific antibodies were detected in one of the two patients with CLTC-ALK positive DLBCL (titre: 1/6750).

In contrast, only one of the 50 children (median age 1.3 years, range 0.1-13.3) with ALK-expressing neuroblastoma (including four cases with *MYC-N* amplification and 11 with chromosome 1p-abnormalities) had a detectable antibody titre, and even this response was weak (1/100). This patient had stage 4 disease; the tumour didn't show *MYC-N* amplification or 1p deletion or somatic *ALK*

mutations. The characteristics of ALK-expression in neuroblastoma differ in many aspects from those of lymphoma patients, which may account for the missing immune response: Transmembrane full-length ALK or variants with point mutations are expressed in neuroblastoma and ALK is usually expressed at far lower levels in neuroblastoma than are detected in ALCL. In addition to the differences in ALK-expression in neuroblastoma, the generation of ALK antibodies in patients with ALK-positive cancer likely also involves other factors. These include the levels of expression of the oncoprotein itself but also other tumour characteristics and the tumour microenvironment. For example, it has been reported that neuroblastoma cells can dampen the immune response by down-regulating expression of HLA^{22,23}. Furthermore, they release soluble inhibitors of the immune system, e.g. gangliosides²⁴. Additionally, since neuroblastomas often arise prenatally further tolerance mechanisms may be active.

Table 1: Anti-ALK antibody titres in patients with ALK positive malignancies other than NPM-ALK-positive ALCL

	ALCL	DLBCL	RMS	NBL	NSCLC
N	20	2	1	50	21
ALK aberration					
Translocation/inversion	20	2	-	-	21
Amplification	-	-	1	-	-
Point mutation	-	-	-	6	-
No specific aberration	-	-	-	44	-
Anti-ALK titre					
0	8	1	0	49	8
≤ 1/750	3	0	1	1	10
1/2250-1/20250	5	1	0	0	2
≥ 1/60750	4	0	0	0	1
Stage*					
1	1	0	0	18	0
2	5	2	0	8	0
3	10	0	0	7	0
4	3	0	1	12	21
4S	N/A	N/A	N/A	5	N/A
Not known outcome	1	-	-	-	-
CCR	15	1	0	32	1
Relapse/alive	5	0	0	6	11 ^a
Relapse/DOD	0	1	1	12	6 ^b
Unknown					3

* Stage definitions according to disease: Lymphoma: St. Jude staging system; Neuroblastoma: INSS.

ALCL, anaplastic large cell lymphoma; DLBCL, diffuse large B cell lymphoma;

N/A, not applicable; CCR, continuous complete remission; DOD, death of disease;

^a stable disease; ^b disease progression

In contrast to the patients with ALK-positive neuroblastoma, the 19 year old patient with alveolar RMS and high expression of full-length ALK clearly produced ALK antibodies. Even though only one RMS patient was assessed this suggests that expression of full length ALK does not preclude the immune response and that anti-ALK immunity

should be systematically analysed in ALK-positive RMS.

The majority of ALK expressing NSCLC patients had progressive disease and 19 of the 21 had undergone prior chemotherapy, in some cases multiple rounds. Prior therapies and long-standing disease might have suppressed the immune response or, at least, lowered the antibody titre as has been observed in ALCL-patients⁹. Therefore, the strength of the immune response might not reflect the titres at initial diagnosis. Despite this limitation, 10 of the 21 ALK-positive NSCLC patients mounted a weak antibody response against ALK ($\leq 1/750$) with eight having no titre at all, two patients displaying moderate titres (>750 - <60750) and one patient having a high titre ($\geq 1/60750$) (Table 1). In addition to the influence of prior therapy on the antibody response, NSCLC, like neuroblastoma, express lower cellular levels of aberrant ALK in comparison to lymphomas²¹. Furthermore, as in neuroblastoma, HLA-down-regulation has been described in NSCLC²⁵. However, these data do indicate that an ALK-specific immune response exists in NSCLC-patients. Our results raise the possibility of boosting the ALK-directed immune response as a therapeutic strategy in patients with ALK-positive NSCLC which is of special interest in light of the still dismal prognosis even with ALK-inhibitor therapy. Furthermore, these data suggest that further studies of ALK antibody titre in NSCLC are warranted, in particular of patients at first disease presentation.

The sera of X-ALK-positive ALCL-patients in this study reacted against NPM-ALK, TPM3-ALK and full-length-ALK transfected COS cells and sera from patients with NPM-ALK- positive ALCL stained cells expressing the TPM3-ALK fusion protein (Supplementary Figure S1). Likewise, sera from ALK-positive NSCLC patients stained both EML4-ALK and NPM-ALK transfectants (data not shown). These data suggest that the auto-antibodies target the ALK-portion of the fusion proteins indicating that the immune response is directed against the embryonal ALK tumour-associated antigen rather than an epitope from the tumour-specific X-ALK fusion site.

None of the 20 healthy control persons had detectable anti-ALK antibodies in their serum. Together with our previous data this equates to more than 100 adults without ALK-expressing tumours (50 healthy at the time of analysis) who do not have a detectable humoral immune response against ALK. These data suggest that unspecific autoimmune reactions against ALK are rare in the absence of ALK-expressing tumours.

In summary, antibody responses against ALK

fusion proteins are not restricted to NPM-ALK-positive ALCL but can be detected in most patients with variant ALK-fusion partners in both ALCL and DLBCL, as well as in one ALK-positive RMS and half of the patients with ALK-positive NSCLC in contrast to the vast majority of patients with ALK-positive neuroblastoma. Our data suggests that inter-individual differences in immune response modifiers may not be the sole determinant as to whether a patient elicits an immune response against ALK. Dependent on the strength and kind of ALK expression (full-length versus fusion protein), tumour cell type and age at tumour development, different immune escape mechanisms may be active. Boosting a pre-existent anti-ALK immune response may be more feasible for patients with ALK-positive NSCLC, lymphomas and rhabdomyosarcomas than for tumours expressing wild-type ALK.

Supplementary Material

Supplementary Figure S1.

<http://www.jcancer.org/v07p1383s1.pdf>

Abbreviations

ALK: anaplastic lymphoma kinase; ALCL: Anaplastic Large Cell Lymphoma; CLTC: clathrin; DLBCL: Diffuse Large B Cell Lymphoma; EML4: echinoderm microtubule associated protein like-4; IHC: immunohistochemistry; NHL: Non-Hodgkin Lymphoma; NPM1: nucleophosmin 1; NSCLC: non-small cell lung carcinoma; RMS: rhabdomyosarcoma; TPM3: Tropomyosin 3; X-ALK: ALK fusion proteins with variant fusion partners.

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Authorship

CDW and WW designed and coordinated the study. FS, MH, SDT, KP and CDW measured

antibody titres and performed molecular analyses. MF, TL, RC, VN, CDW and WW collected samples and provided clinical data. These data were analysed by CDW, SDT, AB and WW. IO performed in depth histological evaluations and collected samples. RS performed and evaluated molecular cytogenetic analysis. CDW, SDT and WW wrote the paper. All authors approved the final version of the manuscript.

Competing Interests

The authors have declared that no competing interest exists.

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