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The anaplastic lymphoma kinase as an oncogene in solid tumors

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TABLE OF CONTENTS

1. ABSTRACT

Twenty years ago anaplastic lymphoma kinase (ALK) was discovered in anaplastic large cell lymphoma (ALCL), but the interest in ALK as an oncogene grew only in recent years when ALK rearrangements were reported as recurrent genetic lesions in lung carcinoma and activating single point mutations were described in neuroblastoma. In this review we will describe the main features of ALK-rearranged solid tumors, with particular emphasis to NSCLC and neuroblastoma. We will discuss the numerous *in vitro* and *in vivo* studies that confirmed ALK as the "driver" oncogene in these tumors and the achievements in clinical settings with ALK inhibitors that validated ALK as a therapeutic target. We will finally end with the description of putative innovative therapeutic approaches that are on going to overcome acquired resistance that invariably occurs in crizotinib treated NSCLC patients or intrinsic resistance to crizotinb therapy reported in neuroblastoma.

2. INTRODUCTION

Anaplastic lymphoma kinase (ALK) is described as the "driver" oncogene in a variety of human cancer, both hematological and solid tumors. ALK was originally cloned and identified in anaplastic large cell lymphoma (ALCL) in 1994 as the result of a recurrent chromosomal translocation, t(2;5; p23;q35) (1). ALK is a receptor tyrosine kinase that belongs to the insulin receptor superfamily. In invertebrates, the role of ALK and its ligands (jelly belly and hesitation behavior-1 (HEN-1)) is clearly defined (2, 3). In *Drosophila melanogaster* ALK is required for survival and is involved in the development of the visual system (4), in visceral muscle patterning (5, 6) and in protecting neural progenitors during nutrient restriction (7). In contrast, in mammals, ALK is considered an orphan receptor and its physiological role is only partially elucidated. Indeed, the biological relevance of two putative ligands for ALK, pleiotrophin and midkine, is still unclear(8, 9). Recently, heparin was proposed to be the ligand for ALK (10). In mammals, ALK is expressed during embryonic and neonatal development in specific regions of the nervous system, whereas in adults it is restricted to some neurons in the central nervous system at barely detectable levels (11-13). In line with these observations, *Alk* knock-out mice are viable and without evident tissue abnormalities showing a very mild phenotype, mostly related to behavioral control (14, 15).

In recent years, ALK rearrangements have been described in other tumors, including non-small cell lung carcinoma (NSCLC), inflammatory myofibroblastic tumor (IMT), diffuse large B-cell lymphoma (DLBCL), renal cell carcinoma (RCC), colon carcinoma, breast and thyroid tumors (16, 17). In addition, activating point mutations and gene amplifications have been reported in neuroblastoma (NB) and anaplastic thyroid cancer (ATC) (18-21) (Figure 1). ALK chromosomal rearrangements generate fusion proteins that invariably contain the

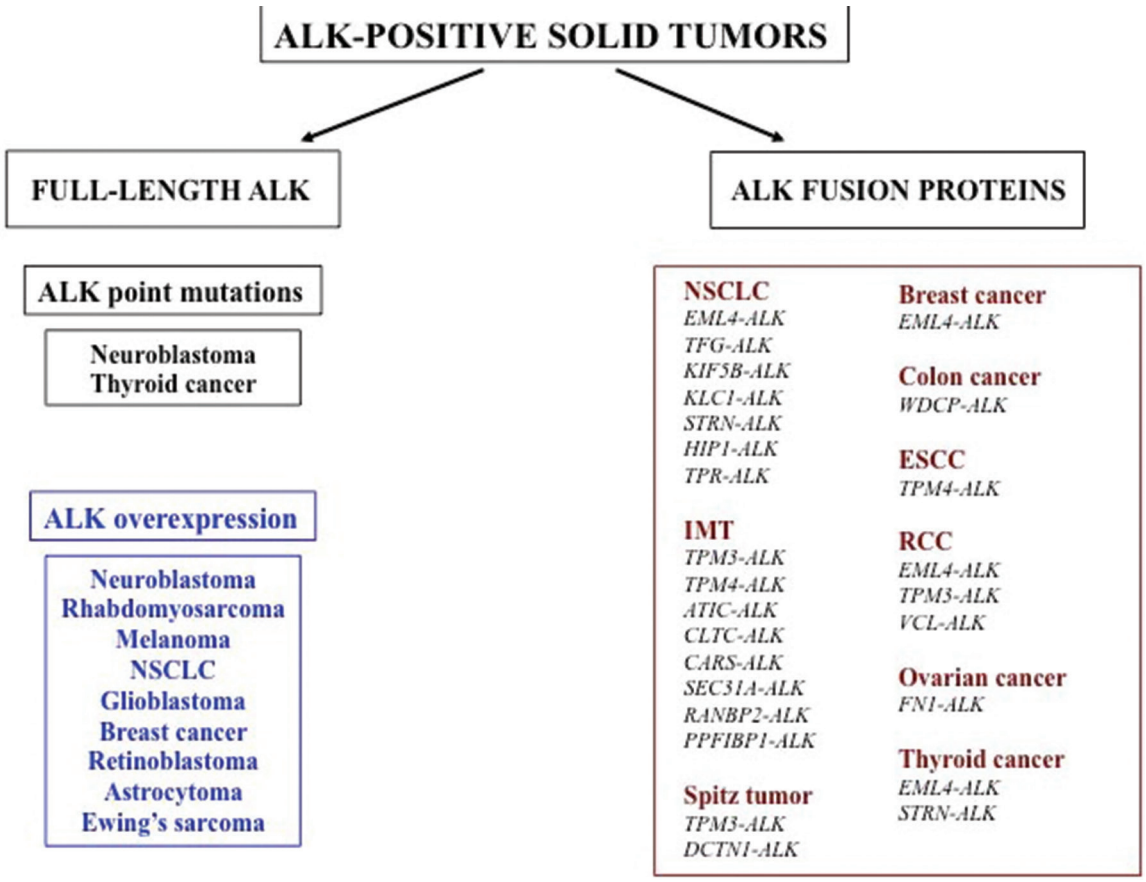

Figure 1. Schematic representation of ALK alterations in solid tumors. NSCLC: non-small cell lung carcinoma; IMT: inflammatory myofibroblastic tumor; RCC: renal cell carcinoma; ESCC: esophageal squamous cell carcinoma

intracytoplasmic signaling portion of ALK fused to a partner that contributes a dimerization domain. Spontaneous homodimerization induces cross-phosphorylation of ALK, and triggers the constitutive tyrosine kinase activity that generates oncogenic signals. The common feature of all ALK fusions is the aberrant activation of ALK downstream signaling and, as reported for ALCL, in the majority of ALK-rearranged tumors the ablation of the ALK signaling leads to growth arrest and cell death (16, 22). The transforming properties and the signaling triggered by ALK have been extensively characterized for NPM-ALK in ALCL. Several downstream pathways are activated by NPM-ALK, with a broad range of signals that lead to increased cell proliferation, survival, motility, and cytoskeletal rearrangements (22). In transgenic mice (23), as well as in lymphoma-derived cell lines, ALK oncogenic signals are mediated by a series of key molecules and pathways, including Stat3 (24, 25), phosphatidyl inositol 3-Kinase (PI3K) (26), Ras/MAPK/ERK, Shp2 (27), p130Cas, (28), PLCγ (29) and Src (30). Similar pathways are activated in ALK-driven lung cancers and neuroblastoma.

3. ALK TRANSLOCATIONS IN SOLID TUMORS

3.1. ALK rearrangements in non-small cell lung cancer

In 2007 ALK rearrangements were identified in a small subset of NSCLC patients as a result of an inversion within chromosome 2, inv 2(2p21;p23) (31, 32). First reports described ALK rearrangements in 6-7% of NSCLC patients, but following studies reported different percentages depending on the population studied, since it is more frequent in the Asiatic population than in the Western population. To date the overall incidence of ALK rearrangements in NSCLC is reported approximately as 5-6% of worldwide NSCLC cases(33). ALK-rearranged NSCLC are mainly adenocarcinoma characterized by the presence of signet-ring cells with abundant intracellular mucin (34, 35) and are prevalent in young non- or light-smoker patients (33, 36). In contrast to EGFR mutations, NSCLC harboring ALK rearrangements do not show any ethnic/racial differences (37). Excluding few rare exceptions, ALK rearrangements are mutually exclusive with other frequent oncogenic mutations found in NSCLC, such as EGFR and KRAS mutations (37-40).

The inversion within the short arm of chromosome 2 generates a fusion between the 5' region of echinoderm-microtubule-associated protein-like 4 (EML4) gene and the 3' region of the ALK gene that creates a new constitutively active ALK fusion protein, EML4-ALK. EML4-ALK contains the entire intracytoplasmic portion of ALK and the N-terminal portion of EML-4 whose coiled-coil domain provides the oligomerization domain for the ligand-independent activation of EML4-ALK (32). To date, multiple variants of EML4-ALK have been described in NSCLC patients due to different breakpoints in the EML4 gene (occurring at exons 2, 6, 13, 14, 15, 18, 20) (33). All EML4-ALK variants are fully active and transforming because they retain the same portion of ALK that includes the kinase domain (33). As described for ALK-rearranged ALCL, additional ALK translocations have also been reported in NSCLC involving different partners, including TRK-fused gene (TFG-ALK), kinesin family member 5B (KIF5B-ALK), kinesin light chain 1 (KLC1-ALK), striatin (STRN-ALK), huntingtin interacting protein 1 (HIP1-ALK) and translocated promoter region (TPR-ALK) (17, 31, 41-44). In contrast to ALCL, all ALK fusions in NSCLC are localized in the cytoplasm where they trigger proliferative and survival downstream pathways (16). Numerous *in vitro* and *in vivo* studies have demonstrated the transforming properties of ALK translocations and the strong addiction to the tyrosine activity of ALK of ALK-rearranged NSCLC. Indeed, NSCLC cells depend on EML4-ALK for growth and survival and are sensitive to ALK inhibition (45, 46) regardless the type of translocation or EML4-ALK variant. However, some *in vitro* observations reported a different sensitivity to ALK inhibitors for some EML4-ALK variants likely related to their protein stability (47). The generation of constitutive and conditional ALK-rearranged transgenic (Tg) mice helped to elucidate the biology of ALK-rearranged NSCLC and provided a useful model for preclinical studies (48, 49). ALK driven NSCLC mouse models developed hundreds of adenocarcinoma nodules in both lungs with a very short latency period, and with 100% penetrance and showed dramatic responses to ALK inhibitors, TAE684 and crizotinb. Recently, the CRISPR/Cas9 technology has been exploited to induce EML4-ALK rearrangement *in vivo* and to generate mouse models of EML4-ALK driven lung cancer (50, 51). All derived tumors expressed the EML4-ALK fusion protein, displayed histopathological features of human ALK-driven NSCLC and responded to crizotinib treatment (50). Interestingly, tumors were strongly positive for the alveolar type II marker surfactant protein C (SP-C) and completely negative for the Clara cell marker CCSP (also known as CC10), thus definitively validating previous mouse models of ALK-rearranged NSCLC in which EML4-ALK expression was forced in type II alveolar epithelial cells by the use of SP-C promoter.

3.2. ALK rearrangements in other solid tumors

The first evidence of ALK involvement in the pathogenesis of IMT dates back to 1999 when the first ALK fusion protein was described in these tumors (52). So far, approximately 50% of IMT harbors ALK rearrangements that lead to different ALK fusion proteins, all sharing the ALK kinase domain, such as TPM3- and TPM4-ALK, ATIC-ALK, CLTC-ALK, CARS-ALK, RANBP2-ALK and SEC31L1-ALK (53-59). In contrast to ALK-driven NSCLC, in which ALK fusions are unique, most of the fusions in IMT have been described in ALCL (60). IMT is frequently diagnosed in young patients and is now categorized as a mesenchymal tumor derived from myofibroblasts, but it was initially considered an "inflammatory pseudo-tumor" because of the presence of a rich inflammatory infiltrate. The discovery of ALK-rearrangements supported the etiology of a low-grade mesenchymal neoplasm, even though the prognostic relevance of ALK rearrangement in IMT is still controversial. Nonetheless ALK-directed therapy is emerging as a highly effective treatment option for a subset of patients with IMT with more aggressive disease, although acquired resistance has been already reported (61, 62).

After the discovery of ALK rearrangements in NSCLC and the promising clinical benefits obtained with the treatment with ALK kinase inhibitors, many efforts have been directed to detect ALK genetic lesions in other solid tumors. To date the list of ALK fusion proteins in other epithelial tumors, although in isolated cases, includes TPM3-ALK, EML4-ALK and vinculin (VCL)-ALK in poor outcome RCC (63-65), EML4-ALK in breast cancer (66), C2orf44-ALK in colon carcinoma, recently reported as WD repeat and coiled coil containing protein (WDCP)-ALK, (39) (67), fibronectin (FN1)-ALK in ovarian cancer (68), EML4-ALK and STRN-ALK in thyroid carcinoma (17), TPM3-ALK and dynactin 1 (DCTN1)-ALK in Spitz tumors(69) and it will likely grow in the future. A recent report on ALK-rearranged anaplastic thyroid cancer demonstrated that STRN-ALK is transforming and tumorigenic *in vivo* and that blockade of its kinase activity with specific ALK inhibitors arrests tumor growth *in vivo* (70). Therefore, although in most of these tumors the role of ALK needs to be fully elucidated, as well as their addiction to ALK, these tumors might benefit from ALK inhibition.

4. ALK POINT MUTATIONS AND OVEREXPRESSION

4.1. Neuroblastoma

Neuroblastoma is the most common extracranial malignant tumor of childhood accounting for 15% of pediatric oncology deaths (71). To date the amplification of the MYCN oncogene on chromosome 2 (2p24) is the most frequent lesion, occurring in about 20-25% of cases and has been used for the stratification of neuroblastoma patients being associated with poor prognosis (72). Recently, the discovery of activating single point mutations of ALK tyrosine kinase receptor in both hereditary and sporadic neuroblastoma allowed

a better stratification of patients and provided the basis for a targeted therapy in these tumors (18-21). Different non-synonymous mutations mostly within the tyrosine kinase domain of ALK have been reported so far in primary tumors (73). The most common mutations were the substitution of the phenylalanine at codon 1174 with different aminoacids (F1174L/C/I) only in sporadic cases, the R1275Q and the F1245C in both familial and sporadic neuroblastoma. Other mutations were detected only in familial cases of neuroblastoma, such as R1192P and G1128A. Interestingly, F1174L was preferentially associated with MYCN amplification in neuroblastoma and was also detected in crizotinib resistant IMT (62, 74). Besides point mutations, ALK overexpression has been detected in the majority of neuroblastoma and has been associated to a worse prognosis in patients (75, 76). Mutations and overexpression induce a constitutive ligand-independent activation of the ALK receptor, with differential transforming ability in various cell types (Schulte et al., 2012). ALK mutations display different phosphorylation status and activate downstream pathways in a mutation-dependent manner, i.e. F1174L preferentially activated STAT3 and AKT, whereas R1275Q efficiently activated AKT1/2 and ERK1/2 (18-21). Knock-down of ALK or inhibition of ALK kinase activity by small molecules in neuroblastoma mutant cell lines led to growth arrest and apoptosis and further proved the “addiction” to mutated ALK of neuroblastoma tumors (18-21, 77). Two different transgenic mouse models of neuroblastoma harboring ALK F1174L mutation under Dbh or Th promoters shed light on the role of ALK mutations in neuroblastoma pathogenesis and provided important clues for the therapeutic testing of ALK inhibitors in neuroblastoma (78, 79). Only one mouse model developed tumors similar to neuroblastoma in presence of ALK F1174L, but with a very long latency and low penetrance (79). In both models, cooperation with MYCN accelerated neuroblastoma formation and led to development of high penetrant and aggressive tumors. The fact that these models developed tumors only when ALKF1174L and MYCN are co-expressed demonstrate that ALK alone is not enough to initiate tumorigenesis in neuroblastoma and need the cooperation with MYCN to develop tumors. Interestingly, both models showed intrinsic resistance to crizotinib treatment in agreement with in vitro assays that reported a different sensitivity to crizotinib for the ALK mutant form F1174L in neuroblastoma cells (78-80). However, using other ALK inhibitors or a combination treatment with mTor inhibitor a partial response in terms of tumor regression was observed thus providing evidence that ALK could be a good target also in MYCN-amplified tumors. A transgenic neuroblastoma model developed in zebrafish confirmed the cooperative role of wild-type or mutated ALK (F1174L) in the pathogenesis of neuroblastoma. As observed in Tg mice, neither ALK wt nor ALK F1174L alone developed tumors (81). In recently generated knock-in mice for both F1174L and R1275Q (murine F1178L and R1279Q, respectively), a prolonged neurogenesis of the sympathetic ganglia has been observed, but not tumor development (82). Also in this context both ALK mutations accelerated and increased MYCN tumor formation and penetrance, with more aggressive behavior in presence of F1174L mutation. These new knock-in models will help to define more precisely the role of ALK in neuroblastoma and will likely represent a better platform for the screening of alternative targeted approaches.

4.2. ALK as an oncogene in anaplastic thyroid cancer and rhabdomyosarcoma

Missense mutations of ALK receptor have been reported in thyroid cancer both in cell lines and primary tumors (83). These mutations increased the kinase activity of ALK and were fully transforming both *in vitro* and *in vivo* assays, but the proof of ALK “addiction” is still missing in this type of tumors. Recently, a recurrent STRN-ALK rearrangement was found in approx. 2% of thyroid cancers, with possibly higher prevalence in poorly differentiated cancers (17, 70, 84).

A recent wide-genome screening identified frequent copy number gain of ALK in rhabdomyosarcoma, an aggressive form of soft tissue sarcoma in children and adolescents with very poor prognosis (85). Copy number gain was associated with high level of ALK protein expression. In contrast to neuroblastoma, ALK copy number gain was not associated with MYCN amplification that is frequently observed in these tumors. ALK copy number gain correlated with more aggressive and metastatic forms of rhabdomyosarcoma, but its clinical relevance as a potential therapeutic target has not yet been assessed (85, 86).

5. TARGETED THERAPIES FOR ALK-REARRANGED SOLID TUMORS

The findings of recurring rearrangements of ALK in NSLCL prompted the development and the use of ALK inhibitors in the clinic. Crizotinib, a potent ATP-competitive inhibitor of c-Met and ALK, which was developed as a MET inhibitor before the identification of EML4-ALK in NSCLC, was rapidly tested in phase I-II clinical trials (87, 88). Due to the exceptionally high rate of clinical responses (tumor regression in nearly 60% of patients), that further supported the “driver” role of ALK fusions in NSCLC harboring ALK rearrangements, crizotinib received in 2011 an accelerated approval from the US Food and Drug Administration (FDA) to treat ALK-rearranged NSCLC. Despite a high rate of response, only a minimal extension in overall survival was achieved in ALK-rearranged NSCLC patients treated with crizotinib because most of the patients developed resistance to the drug (89, 90). Three general mechanisms of acquired drug resistance to crizotinib in NSCLC have been described: (i) activating ALK mutations, spread throughout the tyrosine kinase domain, that affect the drug interaction regions, (ii) ALK

amplification that leads to overexpression of the EML4-ALK fusion and (iii) activation of alternative compensatory signaling pathways, or so-called bypass track activation, where other RTKs or downstream molecules compensate for the inhibited ALK signaling (36, 62, 91-93). Several next-generation ALK inhibitors have been developed to overcome crizotinib resistance in NSCLC patients. The most notable second-generation ALK inhibitors currently in clinical trials are ceritinib (LDK-378, recently approved by FDA), alectinib (CH542802, already approved in Japan), AP26113 (dual ALK and EGFR inhibitor) and PF-06463922(94). Remarkably, in a recent clinical trial ceritinib showed activity in NSCLC patients that relapsed under treatment with crizotinib and prolonged progression free-survival by 7 months, but it is unclear whether the effects were due only to better pharmacodynamics of ceritinib or to a real increased activity against the ALK kinase (95). Nonetheless, acquired resistance to ceritinib has already been described in NSCLC patients (96). Mechanisms of resistance to crizotinib or next-generation ALK inhibitors will be discussed more in details in a parallel review in this issue (Farina et al).

The encouraging results of crizotinib treatment in NSCLC provided a strong rationale for targeting ALK in neuroblastoma. In accordance with the *in vitro* observations, the results of phase I clinical trial in neuroblastoma highlighted the differential sensitivity to ALK kinase inhibition compared to the sensitivity observed in ALK-rearranged tumors (80, 97). Therefore, the intrinsic resistance to ALK inhibitors is a main issue in the treatment of neuroblastoma patients and is emerging as the main mechanism that underlies the lack of tumor responsiveness and relapse in neuroblastoma (98).

Several alternative approaches to treat ALK-driven tumors are currently under investigation, such as next-generation ALK inhibitors or combination therapy aimed to inhibit ALK together with the inhibition of downstream molecules or the so called bypass tracks

A promising treatment in ALK-rearranged NSCLC is represented by inhibition of the heat shock protein-90 (HSP-90) that is a chaperone molecule necessary for the correct folding, stabilization and degradation of many oncogenes, including EML4-ALK. HSP-90 inhibition has shown a therapeutic effect in terms of tumor regression and stabilization of the disease in both mouse models and ALK-rearranged NSCLC patients (49, 99). Importantly, the HSP-90 inhibitor, ganetespib overcame multiple forms of crizotinib resistance *in vitro* consistent with the activity seen in a patient with crizotinib-resistant NSCLC (100). Based on these findings, targeting HSP-90 in ALK-rearranged tumors might be a suitable alternative to small molecule inhibitors in the clinical setting.

Recently, immunotherapy has showed promising efficacy in some solid tumors, including NSCLC (101, 102). In this context, the ALK protein has many features of an ideal tumor oncoantigen that can be exploited for the generation of a cancer vaccine: specificity, strong immunogenicity and absolute requirement for tumor maintenance (22, 103, 104). Indeed, DNA-based ALK vaccine induced a specific immune response in pre-clinical mouse models of ALK-rearranged lymphoma and prevented lymphoma growth (105). More recently, the same ALK vaccine was successfully tested in mouse models of ALK-driven NSCLC and delayed tumor progression in the lungs (Voena *et al*, personal communication). Remarkably, ALK vaccine was highly efficient against the most common crizotinib ALK mutants found in NSCLC. Although not yet tested, these encouraging results in preclinical models of ALK-driven ALCL and NSCLC suggest that ALK-vaccine might also be an optional therapy for ALK+ neuroblastoma.

In addition, ALK-directed immunotherapy with monoclonal antibodies targeting the ALK full-length receptor was reported to induce an antibody-dependent cell mediated cytotoxicity (ADCC) against neuroblastoma cells (106, 107). This approach might represent a therapeutic option for neuroblastoma that express both wild-type or mutated ALK receptor.

6. CONCLUSIONS

The identification of ALK rearrangements in different tumor types have unveiled the ALK locus as a "hot spot" for genetic alterations in the genome of various solid tumors. Future understanding of mechanisms that underlie ALK translocations will likely shed light on ALK mediated tumor initiation and transformation in different tissues. Experimental and clinical evidences throughout the years have validated ALK as therapeutic target in NSCLC and neuroblastoma and have established the basis for ALK-targeted treatment in other ALK-rearranged tumors. Despite the great expectations originated by the identification of a novel specific target for pharmacological therapies in ALK-rearranged tumors, clinical successes have been partial and transient due to acquired or intrinsic drug resistance, urging for the search of additional innovative therapeutic strategies. Future efforts should be then directed to the comprehension of ALK-driven transformation and tumor maintenance in different tumors to clarify ALK oncogene addiction and to find new targets or molecular mechanisms for the development of future therapeutic strategies.

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Abbreviations: ALK: anaplastic lymphoma kinase; NSCLC: non-small cell lung carcinoma; IMT: inflammatory myofibroblastic tumor; DLBCL: diffuse large B-cell lymphoma; RCC: renal cell carcinoma; NB: neuroblastoma; ATC: anaplastic thyroid cancer; HEN-1: hesitation behavior-1; NPM: nucleophosmin; EML4: echinoderm-microtubule-associated protein-like 4; TFG: TRK-fused gene; KIF5B: kinesin family member 5B; KLC1: kinesin light chain 1; STRN: striatin; HIP1: huntingtin interacting protein 1; TPR: translocated promoter region; DCTN1: dynactin 1

Key Words: ALK-rearrangements, Non-Small Cell Lung Cancer, ALK mutations, Neuroblastoma, Solid tumors, Targeted therapy, Review

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