Standard immunohistochemistry efficiently screens for anaplastic lymphoma kinase rearrangements in differentiated thyroid cancer

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Abstract

The anaplastic lymphoma kinase (ALK) gene is frequently rearranged in various types of cancer and is highly responsive to targeted therapeutics. We developed a system to detect rearrangement of ALK in a large group of Korean thyroid cancer patients. We screened 474 malignant or benign thyroid tumor cases to identify ALK fusions. Expression and translocation of the ALK gene were analyzed by immunohistochemistry (IHC), fluorescence in situ hybridization (FISH), and digital multiplexed gene expression (DMGE) analysis in formalin-fixed paraffin-embedded tissues. Four cases of rearrangement of ALK were detected by IHC, and these cases were validated with FISH on 189 samples. On the other hand, DMGE analysis using Nanostring detected three out of four IHC-positive cases. Two rearrangements of ALK were striatin (STRN)–ALK fusions, which were identified by S’RACE analysis. Rearrangements of ALK were found exclusively in v-raf murine sarcoma viral oncogene homolog B (BRAF) WT papillary carcinomas. Given the wide availability and accuracy of IHC for detecting ectopic expression of ALK in the thyroid, we suggest that IHC-based screening can be a practical method for identifying patients with ALK rearrangements in differentiated thyroid cancer.

Key Words

- thyroid cancer
- ALK
- gene rearrangement
- immunohistochemistry

Introduction

Differentiated thyroid cancer (DTC) is a common endocrine malignancy, and its prevalence is increasing worldwide (Chen et al. 2009). The majority of patients with DTC have favorable outcomes after standard therapy that combines surgery, radioactive iodine ablation, and thyroid-stimulating hormone suppression. Nonetheless, in approximately 30% of these treated DTC patients, tumors persist or recur (Mazzaferri & Kloos 2001). In a recent analysis, the DTC cohorts with structurally incomplete response to the initial therapy were found to have a 10-year survival that approached only 50% (Vaisman et al. 2011). As these patients were more likely to have 18F-fluorodeoxyglucose positron-emission-tomography-positive and non-radioactive-iodine-avid disease, the current National Comprehensive Cancer Network guidelines recommend the use of small-molecule...

Identification of the mutations driving DTC prompted the therapeutic use of oral multikinase inhibitors such as sorafenib, which targets rearranged during transfection (RET), v-raf murine sarcoma viral oncogene homolog B (BRAF), and vascular endothelial growth factor receptor (Brose et al. 2014). More recently, rearrangements of anaplastic lymphoma kinase (ALK) that lead to both ectopic expression and constitutive activation of the ALK fusion protein were reported in DTC and anaplastic carcinoma (Godbert et al. 2014, Kelly et al. 2014). Treatment with clinically available ALK inhibitors, such as crizotinib and TAE684, yielded in vitro antitumor efficacy (Kelly et al. 2014) and produced clinical improvement in anecdotal cases (Demeure et al. 2014, Godbert et al. 2014).

To introduce these ALK inhibitors for the clinical treatment of thyroid cancer, the precise frequency of the ALK rearrangement in a wide range of thyroid tumors should be evaluated first. More importantly, a practical diagnostic assay for identifying rearrangements of ALK in thyroid cancer is essential. As is the case with non-small cell lung cancer (NSCLC), immunohistochemistry (IHC) may be a useful tool for screening for rearrangements of ALK in thyroid cancer, as the expression of ALK is extremely limited during adulthood and occurs only in cancer tissues following chromosomal rearrangement (Mino-Kenudson et al. 2010, Shaw et al. 2013).

In this study, we performed comprehensive screening for rearrangements of ALK in a large group of paraffinized thyroid tumor samples using IHC with an ALK-specific antibody. We then confirmed that the expression of ALK protein observed was concordant with rearrangement of ALK using fluorescence in situ hybridization (FISH) and digital multiplexed gene expression (DMGE) analysis. In addition, we reported the discovery of a striatin (STRN)–ALK gene rearrangement in a solid variant, the aggressive histological subtype, and the clinicopathological characterization of ALK-rearranged papillary carcinoma.

**Subjects and methods**

**Case selection**

This study included 474 formalin-fixed paraffin-embedded (FFPE) samples from patients with benign and malignant thyroid tumors collected from 1993 to 2002 (n=256) and from 2010 to 2012 (n=218; Table 1). The samples were collected using a protocol approved by the Institutional Review Board Committee of Seoul National University Hospital.

Two cores of 2.0-mm tissue were obtained from the most representative area of the individual cases, and a tissue microarray block was constructed as described previously (Garcia-Gonzalez et al. 2005). The BRAFV600E mutation was prescreened in samples of papillary carcinomas using the PCR, restriction fragment length polymorphism analysis, and direct sequencing. We also included 29 matched fresh-frozen samples of WT BRAF papillary carcinoma for detailed genomic analysis. Medical records of the cases with ALK rearrangements were reviewed to extract data including demographic characteristics, histological subtype, tumor–nodes–metastases (TNM) staging according to the Seventh American Joint Committee on Cancer (Edge et al. 2010), and clinical course.

**Table 1** Summary of study samples screened for rearrangement of ALK

<table>
<thead>
<tr>
<th>Histology</th>
<th>Sample type</th>
<th>ALK IHC</th>
<th>ALK FISH</th>
<th>ALK Nanostring</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Positive (n)</td>
<td>Total (n)</td>
<td>Positive (n)</td>
</tr>
<tr>
<td>Papillary carcinoma</td>
<td>Fresh-frozen and FFPE</td>
<td>1</td>
<td>29</td>
<td>1</td>
</tr>
<tr>
<td>WT BRAF</td>
<td>FFPE</td>
<td>3</td>
<td>233</td>
<td>3</td>
</tr>
<tr>
<td>BRAFV600E mutant</td>
<td>FFPE</td>
<td>0</td>
<td>79</td>
<td>0</td>
</tr>
<tr>
<td>Follicular carcinoma</td>
<td>FFPE</td>
<td>0</td>
<td>25</td>
<td>0</td>
</tr>
<tr>
<td>Hurthle cell carcinoma</td>
<td>FFPE</td>
<td>0</td>
<td>9</td>
<td>0</td>
</tr>
<tr>
<td>Anaplastic carcinoma</td>
<td>FFPE</td>
<td>0</td>
<td>17</td>
<td>0</td>
</tr>
<tr>
<td>Benign thyroid tumor</td>
<td>FFPE</td>
<td>0</td>
<td>57</td>
<td>0</td>
</tr>
<tr>
<td>Follicular adenoma</td>
<td>FFPE</td>
<td>0</td>
<td>25</td>
<td>0</td>
</tr>
<tr>
<td>Hurthle cell adenoma</td>
<td>FFPE</td>
<td>0</td>
<td>25</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>4</td>
<td>474</td>
<td>4</td>
</tr>
</tbody>
</table>

FFPE, formalin-fixed paraffin-embedded; ND, not done.

*The Nanostring result for patient 4 seemed to be a false negative because the remaining amount of FFPE sample was insufficient after FISH and IHC analyses.
Immunohistochemistry

IHC staining was performed in FFPE tissue microarray sections that were 4 μm thick using an automated immuno-stainer (Leica Microsystems, Milton Keynes, UK). Briefly, the slides were heated for 20 min at 100 °C in Epitope retrieval solution, pH 9.0 (Leica Microsystems). The slides were then incubated with a monoclonal mouse anti-human ALK antibody (Novocastra, Newcastle Upon Tyne, UK) at a dilution of 1:25. This antibody was raised against a C-terminal portion of the tyrosine kinase domain of ALK and was intended for the qualitative identification of ALK molecules in paraffin sections by light microscopy. Staining intensity was scored as 0 (no staining), 1+ (weak cytoplasmic staining without any background staining), and 2+ (strong cytoplasmic staining). Tumors with 1+ or 2+ expression in more than 10% of the tumor cells were deemed positive for ALK protein expression (Yi et al. 2011, Park et al. 2012). For ALK IHC-positive cases, we subsequently performed IHC using an antibody against phosphorylated ALK (phosphor Y1507, Abcam, Cambridge, MA, USA) at a dilution of 1:100.

Fluorescence in situ hybridization

ALK rearrangements in FFPE tumor tissues were detected by FISH analysis using a break apart probe specific for the ALK locus (Vysis, Abbott Molecular), according to the manufacturer’s instructions. Cases were defined as positive by FISH when tumors harbored split signals in more than 15% of cells or an isolated red (3’) signal in tumor cells as described previously (Kim et al. 2013, Kelly et al. 2014).

RNA isolation from FFPE and fresh-frozen samples

Six sections from archived FFPE blocks were used for RNA extraction using a High Pure FFPE RNA Isolation kit (Roche). AllPrep DNA/RNA Minikit (Qiagen) was used for RNA isolation from the fresh-frozen biopsy samples. The extracted RNA was quantified and its quality was assessed using a Nanodrop 8000 (Thermo Scientific, Waltham, MA, USA) and a Bioanalyzer 2100 (Agilent, Santa Clara, CA, USA).

5’ RACE

To determine the N-terminal ALK partner, a SMARTer RACE cDNA Amplification Kit (Clontech) was used according to the manufacturer’s instructions (Supplementary Fig. 1, see section on supplementary data given at the end of this article). Touchdown PCR was performed using LA HS Taq DNA polymerase (Clontech) with the following primers: ALK–gene-specific primer 1 (5’-CCGCCCATGAGCTCCACGGATGAAC-3’), Universal Primer A Mix, ALK–gene-specific primer 2 (5’-CAGGGCTTCCCAGGAAATCCAGT-3’), and Nested Universal Primer A. Amplified products were ligated into the pGEM-T easy Vector (Promega) and subjected to nucleotide sequencing (Macrogen, Seoul, Korea).

RT-PCR for STRN–ALK

To verify the presence of STRN–ALK fusion transcripts, cDNA was synthesized from total RNA of fresh-frozen and FFPE samples using the ReverTra Ace qPCR RT Kit (Toyobo, Osaka, Japan). The PCR was performed with 35 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 1 min, and elongation at 72 °C for 1 min. The specific fusion primers for STRN–ALK were as follows: STRN exon 3 forward primer (5’-CGGACAGAATTTAACGG-3’) and ALK exon 20 reverse primer (5’-TGCCAGCAGCAGTATGGT-3’).

Nanostring nCounter analysis

According to the manufacturer’s instructions (Nanostring Technologies, Inc., Seattle, WA, USA), the nCounter assay was performed in order to confirm potential ‘fusion calls’ in a total of 32 FFPE samples of papillary carcinoma. In brief, 200 ng of total RNA from FFPE and selected fresh-frozen samples were hybirdized to nCounter reporter probe sets (Supplementary Table 1 and Supplementary Fig. 2, see section on supplementary data given at the end of this article) for 16 h at 65 °C. The hybridized samples were transferred to the nCounter Sample Prep station, and the reporter signals were visualized using a digital analyzer and normalized using the nSolver analysis software version 1. The background threshold was calculated from negative controls for a potential ‘fusion call’. The calculation was performed as follows: ALK3'/ALK3'-Max(A5, B), where E5 is the geometric mean of ALK 3’ probe expression, A5 is the average of ALK 5’ probe expression, and B is the background threshold as defined previously (Lira et al. 2013, Fang et al. 2014). The thresholds for RET and fusion probes were calculated in a similar manner as described previously (Lira et al. 2013, Fang et al. 2014).

Results

Identification of STRN–ALK fusion in fresh tissue samples

To search for ALK rearrangements in thyroid cancer, we first examined FFPE samples from 29 cases of papillary
cancer with WT BRAF that also had fresh frozen tissue available. Of these, one case (patient 1) showed strong cytoplasmic staining for ALK by IHC (Fig. 1A), indicative of abnormal expression due to rearrangement of ALK. The break apart FISH assay confirmed rearrangement of ALK through visual splitting of one pair of red and green signals (Fig. 1B). The remaining 28 samples exhibited fused signals (Fig. 2H), indicating normal ALK status.

We then performed S’ RACE to identify the ALK fusion partner in both fresh-frozen and FFPE samples from patient 1. As shown in Fig. 1C, exon 3 of STRN, was fused with exon 20 of ALK, generating a STRN–ALK fusion. The fusion was further verified by RT-PCR with specific primers for 5’-STRN and 3’-ALK (Fig. 1D). This fusion event has been reported recently in a study that examined gene rearrangements in thyroid cancer by paired-end RNA sequencing (Kelly et al. 2014).

**Patient screening using ALK IHC in archival paraffinized samples**

To determine the frequency of rearrangements of ALK in thyroid tumors, we screened a tissue microarray of FFPE samples archived from 1993 to 2002. The samples consisted of 123 papillary carcinomas (44 BRAF WT, 79 BRAFV600E mutants), 25 follicular carcinomas, nine Hurthle cell carcinomas, 17 anaplastic carcinomas, and 82 benign thyroid tumors (Table 1). A positive result for ALK screening by IHC was not obtained for any of these cases. To enrich for ALK-positive cases, the array of BRAF WT papillary carcinomas archived consecutively from 2010 to 2012 was further screened. Among the 189 FFPE samples, three cases showed cytoplasmic ALK localization in IHC (Fig. 2). ALK IHC was performed on those 189 FFPE array samples including the three samples that were positive for ALK by IHC. The results showed break apart or isolated red (3’) signals for only these three, corroborating the results for IHC (Fig. 2). The S’ RACE analysis of the FFPE samples revealed one additional case (patient 2) with an STRN–ALK rearrangement but failed to determine specific fusion partners for the remaining two cases.

Subsequent IHC using an antiphosphorylated ALK antibody revealed activated expression of ALK protein in the four cases with rearranged ALK (Supplementary Fig. 3, see section on supplementary data given at the end of this article). As thyroid cancer commonly harbors RET/PTC translocations (Schlumberger 1998, Nikiforov & Nikiforova 2011), we also performed RET FISH on the ALK-rearranged cases; however, no rearrangements of RET were detected (data not shown), indicating exclusiveness. The overall frequency of rearrangements of ALK was 1.5% in the BRAF WT papillary carcinoma samples (four of 262). Other types of thyroid cancer, including BRAFV600E mutant papillary carcinoma and benign thyroid tumors, did not harbor the ALK rearrangement.

**Comparison between Nanostring and FISH for the detection of rearrangement of ALK**

To explore an alternative screening method for the detection of ALK rearrangement, we employed the Nanostring nCounter assay (Suehara et al. 2012). This assay allows simultaneous detection of multiple translocations using limited amounts of archival paraffinized samples. For parallel comparison with other methods, a total of 32 FFPE samples of BRAF WT papillary carcinoma cases, including the four ALK-rearranged cases identified by IHC/FISH, were subjected to the Nanostring assay (Table 1). Using multiplexed probes to target either the S’ or the 3’ exons of ALK, we assessed the 3’/S’ exon
imbalance. With a $3’/5’$ ratio of greater than two as a cut-off, three samples were deemed positive for ALK rearrangements. One discordant result (positive by IHC/FISH and negative by Nanostring) was obtained for patient 4, and this sample included an insufficient amount of RNA due to a limited quantity of sample, indicating that the discordance arose from sampling rather than the platform. For the remaining 28 patients, we confirmed the expression of native ALK (Table 2, Fig. 3 and Supplementary Table 2, see section on supplementary data given at the end of this article). We also assessed the presence of rearrangements of RET by Nanostring using probes that target the RET exonic imbalance or specific RET/PTC fusion junction. Out of 32 FFPE samples of BRAF WT papillary carcinoma, only one case exhibited the RET/PTC rearrangement (3.1%, Supplementary Fig. 4). Although the number of samples was limited, these results indicate that ALK and RET translocations are mutually exclusive as in the case of rearrangement of ALK and BRAF V600E mutation (Kelly et al., 2014, Perot et al. 2014).

Clinical characteristics of patients with papillary carcinomas that harbor rearrangements of ALK

In this series of patients with papillary carcinomas with rearrangements of ALK, a distinct preference in terms of demographic factors or histological subtypes was not found (Table 2). All patients denied having a history of neck irradiation. Notably, the STRN–ALK rearrangement was found in a young patient with solid variant papillary carcinoma. Patients with this variant are known to have a worse prognosis (Nikiforov et al. 2001). Patient 2, on the other hand, had small follicular variant papillary carcinoma, which is known to have a better prognosis. All four patients with rearrangement of ALK showed favorable responses to the initial surgery with or without radioactive iodine ablation therapy.

Discussion

Analysis of thyroid tumor tissue archives revealed that approximately 1.5% of BRAF WT papillary carcinomas harbor rearrangements of ALK. Our report is the largest study to date to report the frequency of rearrangements of ALK in thyroid cancer. An estimated 40 568 and 62 980 new cases of thyroid cancer each year are diagnosed in Korea (Korea Central Cancer Registry 2011) and the USA (Siegel et al. 2014) respectively. As a result, we extrapolated the number of patients with papillary carcinoma with rearrangements of ALK to be 192 in Korea and to be 468 in the USA, assuming that 90% of all thyroid cancers are papillary carcinoma and that the BRAF V600E mutation rate of each country is 65 and 45% respectively (Kim et al. 2012). ALK is involved in the initiation and progression of many different cancer types (Mano 2012, Hallberg & Palmer 2013), including lymphoma, neuroblastoma, and NSCLC. Preclinical studies in thyroid cancer patients (Kelly et al. 2014), indicated that rearrangements of ALK
in thyroid cancers may also be sensitive to ALK inhibitors. Therefore, we expect that tumors with rearrangement of ALK will become a unique, targetable subset of thyroid cancer and that identification of this emerging biomarker will have an effect on the diagnosis and treatment of patients with advanced DTC.

Diagnostic tests for identifying rearrangements of ALK are important in clinical settings, as such tests can identify those patients who will probably benefit from a targeted therapy. Traditionally, FISH analysis using probes flanking the ALK locus has been used for the identification of this rearrangement (Villamor et al. 2008); however, FISH may not be the optimal technique for routine practice due to the high cost and need for technical expertise. The break apart signal patterns resulting from the intrachromosomal deletion (STRN–ALK) and inversion (EML4–ALK) are often subtle and can easily be missed by this method (Mino-Kenudson et al. 2010). Alternatively, Japanese researchers extracted RNA from stored FFPE samples and used highly sensitive RT-PCR in conjunction with S’ RACE to detect the presence of a high frequency of EML4–ALK rearrangements in papillary carcinomas from atomic bomb survivors (Hamatani et al. 2012).

In this study, papillary carcinomas with ALK rearrangement invariably expressed an ALK protein that could readily be identified in the paraffinized samples using IHC with an ALK MAB. Indeed, no false FISH-negative cases were present in the four IHC-positive cases. As the rearrangement of ALK results in loss of the transmembrane domain of ALK (Shaw et al. 2013), ALK IHC showed cytoplasmic staining in all of the rearranged cases. Given the wider availability and accuracy for detection of ectopic expression of ALK in the thyroid, we suggest that IHC-based screening should be used as a method for identifying thyroid cancer patients with rearrangements of ALK. As rearrangements of ALK represent a potential therapeutic target and FISH is a validated method for the detection of actual fusions that correlate with the response to treatment, we recommend the use of IHC as a screening procedure and the use of FISH for the final confirmation of rearrangement of ALK. If a FISH assay is not available, the critical issue in clinical settings would be to quickly determine whether expressed ALK protein is activated or not, as some papillary carcinomas express WT ALK. In that case, additional use of antibodies against phosphorylated ALK would strengthen the IHC-based screening results, as

<table>
<thead>
<tr>
<th>Table 2 Clinical and histological details of the four patients with papillary carcinoma with rearrangements of ALK</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age (years)</strong></td>
</tr>
<tr>
<td><strong>Sex</strong></td>
</tr>
<tr>
<td><strong>History of neck irradiation</strong></td>
</tr>
<tr>
<td><strong>Preoperative cytology</strong></td>
</tr>
<tr>
<td><strong>Surgery</strong></td>
</tr>
<tr>
<td><strong>Tumor size (cm)</strong></td>
</tr>
<tr>
<td><strong>Histological subtype</strong></td>
</tr>
<tr>
<td><strong>Extrathyroidal extension</strong></td>
</tr>
<tr>
<td><strong>Multilocality</strong></td>
</tr>
<tr>
<td><strong>Lymph node metastasis</strong></td>
</tr>
<tr>
<td><strong>Other pathology</strong></td>
</tr>
<tr>
<td><strong>TNM staging</strong></td>
</tr>
<tr>
<td><strong>BRAF</strong></td>
</tr>
<tr>
<td><strong>Adjuvant radioactive iodine ablation (cumulative dose)</strong></td>
</tr>
<tr>
<td><strong>Follow-up status (duration)</strong></td>
</tr>
<tr>
<td><strong>Tests for ALK rearrangement</strong></td>
</tr>
<tr>
<td></td>
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</tbody>
</table>

CND, central neck dissection; MRND, modified radical neck dissection; ND, not done; NED, no evidence of disease; TT, total thyroidectomy. *The Nanostring result for patient 4 seemed to be a false negative, because the remaining amount of FFPE sample was insufficient after FISH and IHC analyses.

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A relationship between radiation exposure and EML4–ALK rearrangement has been suggested in a previous report (Hamatani et al. 2012); however, subsequent studies, including ours, failed to identify an association between radiation exposure and rearrangement of ALK (Leeman-Neill et al. 2013, Kelly et al. 2014). The mutual exclusiveness of rearrangement of ALK and the BRAFV600E mutation as well as with rearrangement of RET in this study is consistent with previous reports (Kelly et al. 2014, Perot et al. 2014) and supports the concept that rearrangement of ALK is an independent driver in papillary carcinoma.

Given the high potential of receptor tyrosine kinases as therapeutic targets, systemic approaches are necessary in the future to identify activated kinase fusions. In our study, we explored Nanostring nCounter analysis as a systemic approach that can be multiplexed with as many as 800 different probes. In the Nanostring analysis, the specificity for ALK fusion was 100%, indicating its utility as a multikinase screening platform for thyroid cancer. Targeted next-generation sequencing (NGS) may also serve as an alternative multiscreening method (MacConaill 2013, Nikiforov et al. 2014). In recent years, NGS techniques capable of sequencing all known oncogenic drivers, including gene rearrangements, have been actively incorporated into clinical practice, and numerous clinical trials have been performed with the molecular targets identified. In thyroid cancer, BRAFV600E mutation and RET kinase fusions are known to be frequent genomic alterations (Nikiforov & Nikiforova 2011), indicating that these kinases can serve as therapeutic targets in the face of failure of conventional treatment (Brose et al. 2014, www.clinicaltrial.gov; identifier number: NCT01876784).

The routine screening for rearrangement of ALK for postoperative risk stratification in DTC may not offer a clinical benefit considering the low frequency of rearrangements of ALK and the lack of a clear association between rearrangement of ALK and poor clinical outcome of thyroid cancer. As there is no indication for targeted therapy in patients that showed good response to conventional treatment, the usefulness of screening of ALK with respect to the clinical outcome of DTC should be sought in situations involving patients with advanced disease that is not manageable by surgery or administration of radioactive iodine. Interestingly, a recent prospective screening study has revealed that patients with rearrangements of ALK in advanced NSCLC had improved overall survival with an effective targeted therapy than patients without rearrangement of ALK after failure of at least one line of chemotherapy (Fallet et al. 2014).

**Figure 3**

Nanostring results for ALK rearrangement in 32 paraffinized samples of papillary carcinoma are documented as 3′:5′ ratio. The background threshold is indicated by a horizontal line. P1–4 indicates patients 1–4 who have ALK rearrangements. Other dots indicate patients with native ALK. A full colour version of this figure is available via http://dx.doi.org/10.1530/ERC-14-0467.
A considerable body of evidence now supports the molecular phenotype-stratified approach to cancer care (Willyard 2013). In patients with radioactive-iodine-refractory thyroid cancer, the use of practical diagnostic methodology such as IHC to identify patients with rearrangements of ALK is important not only for its immediate influence on care of individual patients but also for the recruitment of a responsive subgroup into a ‘basket’ trial.

Supplementary data
This is linked to the online version of the paper at http://dx.doi.org/10.1530/ERC-14-0467.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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