## Identification of ALK Gene Alterations in Urothelial Carcinoma

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Identification of ALK Gene Alterations in Urothelial Carcinoma

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Abstract

Background: Anaplastic lymphoma kinase (ALK) genomic alterations have emerged as a potent predictor of benefit from treatment with ALK inhibitors in several cancers. Currently, there is no information about ALK gene alterations in urothelial carcinoma (UC) and its correlation with clinical or pathologic features and outcome.

Methods: Samples from patients with advanced UC and correlative clinical data were collected. Genomic imbalances were investigated by array comparative genomic hybridization (aCGH). ALK gene status was evaluated by fluorescence in situ hybridization (FISH). ALK expression was assessed by immunohistochemistry (IHC) and high-throughput mutation analysis with Oncomap 3 platform. Next generation sequencing was performed using Illumina Genome Analyzer Iix, and Illumina HiSeq 2000 in the FISH positive case.

Results: 70 of 96 patients had tissue available for all the tests performed. Arm level copy number gains at chromosome 2 were identified in 17 (24%) patients. Minor copy number alterations (CNAs) in the proximity of ALK locus were found in 3 patients by aCGH. By FISH analysis, one of these samples had a deletion of the 5’ALK. Whole genome next generation sequencing was inconclusive to confirm the deletion at the level of the ALK gene at the coverage level used. We did not observe an association between ALK CNA and overall survival, ECOG PS, or development of visceral disease.

Conclusions: ALK genomic alterations are rare and probably without prognostic implications in UC. The potential for testing ALK inhibitors in UC merits further investigation but might be restricted to the identification of an enriched population.

Introduction

Urothelial carcinoma (UC) accounts for 15,210 cancer deaths per year in the United States [1]. Five-year survival for patients with muscle invasive (T2) disease or greater is only 50%.

Advanced UC of the bladder is often associated with mutations and multiple somatic copy number alterations [2]. Comparative genomic hybridization studies of bladder carcinomas and cell lines have revealed a number of recurrent genetic aberrations including amplifications or gains on 8q22-24, 11q13, 17q21, and losses on chromosomes 9, 8p22-23, and 17p6-9 [3,4]. In several clinical cohorts, some of these genomic alterations have also been associated with pathological stage and outcome [5].

In the recent years, potential new targets for treatment intervention have been described in urothelial tumors. The identification of driving genomic alterations as mutations even if occurring in only a small subset of bladder cancer patients, may lead to the development of patient-specific therapies as has been the case of the recently described mutations in TSC1 predicting response to mTOR inhibitors like everolimus [6–8]. Another example is the PIK3CA gene, mutated in up to 26% of cases in the
series by Ross and colleagues that may predict sensitivity to PI3KCA/mTOR inhibitors [9].

The ALK (anaplastic lymphoma Kinase) inhibitor crizotinib, has recently shown high efficacy in the treatment of patients with non-small cell lung cancer (NSCLC) with ALK translocation which is present in about 4–7% of the tumors [10–12]. In a phase I study of NSCLC patients with an ALK translocation, the response rate was 57% independent of performance status or number of previous treatments with a 70% probability of progression free survival at 6 months [13]. In several other tumor types besides lung cancer, ALK genomic alterations have been identified as potential oncogenic drivers, meaning that cancers in different organs can be targeted for treatment with ALK inhibitors regardless of their cell of origin.

In UC, ALK copy number gain, amplification, translocations, mutations, or expression have not been characterized. We therefore investigated ALK protein expression and underlying genetic aberrations in a cohort of patients who received chemotherapy in the setting of metastatic disease, focusing on clinical and prognostic implications.

In the present study we show that ALK genomic alterations, such as copy number alterations (CNA) and deletions, occur in UC. Additionally, we attempted to identify the impact of these alterations with clinical and outcome features.

**Material and Methods**

**Patients**

This project was approved by the local ethics committee (CEIC-IMAS) at Hospital del Mar, and by the Dana-Farber/Harvard Cancer Center (DF/HCC) institutional review board (IRB). Because the majority of patients were died at the time of collecting samples, a waiver of consent was requested and given from IRB of DF/HCC for all participants (requiring complete deidentification of the samples prior the analysis).

A cohort of 96 patients, with metastatic UC treated with platinum-based combination was identified. All patients underwent several treatment regimens, all containing gemcitabine and a platinum compound, with some patients receiving additional paclitaxel as well. Patient clinical data was collected. The final cohort included 70 patients (52 males, 18 females) with available clinical data and sufficient tissue samples to conduct all the genomic studies.

**Tumor Samples**

The analysis was performed in formalin-fixed paraffin embedded (FFPE) tissue from UC of the urinary tract. Other molecular studies have been performed and reported in these samples in order to characterize the biology of UC [14]. The specimens were retrospectively retrieved from the pathology archive at Hospital del Mar and Mar Biobank in Barcelona, Spain. Slides were reviewed separately by two genitourinary specialist pathologists (MS, DB). All patients had high grade transitional cell carcinoma and no other histological variant was included in this study. Tumor areas were evaluated by a single pathologist (DB) and tumor bearing 0.6 mm cores were punched for DNA extraction and/or tissue microarray (TMA) construction.

**ALK analysis**

ALK genomic alterations were evaluated by array comparative genomic hybridization (aCGH), fluorescence in situ hybridization (FISH), immunohistochemistry (IHC), mass spectrometry mutation analysis and next-generation sequencing. Description of methods can be found in the appendix (Methods S1).

**Statistical analysis**

Statistical analysis of clinical data and molecular features was carried out with SAS version 9.2 (SAS Institute Inc, Cary, NC). Patient and clinical characteristics were summarized as number and percentages for categorical variables and median and interquartile ranges for continuous variables. Overall survival (OS) was defined from the date patients received first line chemotherapy for advanced disease until date of death or censored on the last known alive date. ALK copy number alteration was defined as having more than a 4 fold change [15]. Fisher exact test was used to assess the associations of ALK copy number alteration with ECOG PS and whether patients developed visceral disease. Cox proportional hazard model was used to assess the associations of ALK copy number alteration and overall survival in both univariate and multivariate analyses. Kaplan-Meier estimate was used to summarize median overall survival. All the statistical tests were conducted at the two-sided 0.05 level of significance.

**Results**

The median OS was 12 months with 45 patients deceased at the time of analysis, with a median follow-up of 23 months. Table 1 summarizes patient and clinical characteristic for the entire cohort as well as for patients with more than 4 fold copy number gain in the FISH analysis.

**Recurrent chromosomal gains and losses by aCGH**

Analysis by aCGH of the 70 patients included in the study identified 95 focal and 21 broad (identified as >50% of the chromosome arm) events. The results of the broad alteration analysis were largely consistent with the current literature [16–18]. We observed frequent losses of chromosomes 9q (43%), 8p (69%), 9 (p: 48%; q: 41%), 10q (41%), 11p (49%), 17p (51%), and 22q (40%) and recurrent gains of chromosomes 3q (46%), 5p (48%), 8q (48%), 19q (34%), and 20 (60%). Three specimens out of 70 harbored minor non-significant alterations (log2 ratio 0–0.8) in chromosome 2, where ALK gene locus is located. This encouraged us to conduct a more in-depth search of ALK genomic alterations and to further characterize the 5’ALK deletion seen by FISH in one patient.

**FISH analysis of ALK gene/copy number gains**

To further characterize genomic imbalances on chromosome 2, all samples underwent FISH analysis. One case presented a deletion of the green signal (5’ALK), centromeric to the ALK gene, and also had gain of the ALK gene fusion signals and 3’ALK signal (Figures 1 and 2). This FISH pattern was interpreted as an ALK atypical rearrangement as has been described in ALK positive NSCLC because a single orange (3’ALK) signal was seen [19]. In these cases it is assumed that the deletion is the result of translocation. Analyses of EML4 as well as other known fusion partners such as TGF and KIF5 were performed without finding any translocation of these genes. Even so, it is possible that the deletion does not cause the ALK translocation and other molecular techniques need to be applied to further characterize the FISH findings.

**ALK gene copy number gains and amplification**

Two patients presented amplification of ALK. 90% of samples showed ALK copy number gain due to polysomy of chromosome 2. All of them had 5 to 6 copies of CEP2 except one case with high polysomy. Among 70 urothelial tumors, 7 (10%) demonstrated 2F signals (2 intact ALK loci), 46 (65.7%) had 3–4F signals present, and 17 (24.3%) had ≥5F signals (range 5F–11F; median 6F) in >10% of nuclei (Table 2). The associations of
### Table 1. Patients and Clinical Characteristics.

<table>
<thead>
<tr>
<th></th>
<th>All patients (N = 70)</th>
<th>Patients with copy number alteration (N = 17)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>N % or median (q1, q3)</td>
<td>N % or median (q1, q3)</td>
</tr>
<tr>
<td>Age</td>
<td>61 63 (54, 68)</td>
<td>15 66 (58, 68)</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>52 74%</td>
<td>15 88%</td>
</tr>
<tr>
<td>Female</td>
<td>18 16%</td>
<td>2 12%</td>
</tr>
<tr>
<td>ECOG PS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>22 31%</td>
<td>4 24%</td>
</tr>
<tr>
<td>1, 2</td>
<td>48 69%</td>
<td>13 76%</td>
</tr>
<tr>
<td>Visceral diseases</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>41 59%</td>
<td>7 41%</td>
</tr>
<tr>
<td>Yes</td>
<td>29 41%</td>
<td>10 59%</td>
</tr>
<tr>
<td>Pathological stage</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stage 0 (Ta)</td>
<td>5 7%</td>
<td>2 12%</td>
</tr>
<tr>
<td>Stage I (T1)</td>
<td>5 7%</td>
<td>0 0%</td>
</tr>
<tr>
<td>Stage II (T2)</td>
<td>36 51%</td>
<td>8 47%</td>
</tr>
<tr>
<td>Stage III (T3, T4)</td>
<td>22 31%</td>
<td>7 41%</td>
</tr>
<tr>
<td>Stage IV (L, M)</td>
<td>1 1%</td>
<td>0 0%</td>
</tr>
<tr>
<td>Missing</td>
<td>1 1%</td>
<td>0 0%</td>
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doi:10.1371/journal.pone.0103325.t001

Figure 1. 1298case – FISH + for ALK variant (green probe missing).
doi:10.1371/journal.pone.0103325.g001
Figure 2. 1298case –FISH copy gain (a) & amplified (b).
doi:10.1371/journal.pone.0103325.g002
ALK copy number alteration with ECOG PS, visceral disease, and OS are summarized in Tables 3 and 4. No significant association between ALK copy number alteration and clinical features or overall survival was observed (Figure 3).

Comparison of ALK gene copy number gains to clinical and pathological features for the 70 patients are summarized in Table 1. There were no differences between ALK gene copy number gains and clinical features in all the subgroups (2F, 3–4F and ≥5F). OS rates for patients with 2F+3–4F and ≥5F were 12 and 16 months respectively. There was no statistically significant difference between these groups (Figure 3).

ALK protein expression by immunohistochemistry

To further characterize whether ALK protein expression was affected, immunohistochemistry analysis of all FFPE samples was performed using the Cell Signaling antibody. Immunohistochemistry staining was negative in the tumor with ALK FISH positive test. Similarly, among tumors with ALK gene copy gain or amplification, ALK protein expression was not detected. None of the tumors classified as ALK negative by FISH showed ALK protein expression by immunohistochemistry.

High-throughput mutational analysis using Oncomap

To have more accurate information on genetic alteration in these UC samples, mass spectrometry mutation analysis was also performed for all samples. Ninety-six samples were submitted for OncoMap: 87/96 (91%) passed all quality control steps, 79% (69/87) passing samples harbored candidate mutations. In total, 150 candidate mutation calls were made across 47 genes. Overall, 39% (58/150) of candidate mutations in passing samples were conservative and 61% (92/150) were aggressive. No mutations in ALK were found using this platform. ALK P496L candidate mutation was found in one of the sample but was not confirmed with HME.

Next-generation sequencing of ALK gene

Since FISH technique gives no information of the specific sequence and the exact size of the deleted fragment in ALK, directed analysis of ALK gene was performed by next generation sequencing (Illumina). Analysis of the region containing P496 only showed base changes at rates below 1%, reflecting the expected sequencing error rate. Thus, only the wild-type sequence for the position P496 was detected and no mutations on ALK were detected by this technique.

We then extended the search space to the centromere with the intention to explore potential deletions according to FISH results. In the new analysis performed on the FISH positive patient, one read of a pair should match within the ALK locus, 29.37 Mb - 32 Mb, and one read should match at some place towards the centromere (>10 kb up to position 93.3 Mb). However, at the coverage level used no deletions could be confirmed with this approach.

**Table 2. Copy Number Alteration.**

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<td>7</td>
<td>10</td>
</tr>
<tr>
<td>3–4F</td>
<td>46</td>
<td>66</td>
</tr>
<tr>
<td>≥5F</td>
<td>17</td>
<td>24</td>
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doi:10.1371/journal.pone.0103325.t002
Table 3. Association of ALK copy number alteration with ECOG PS and visceral disease.

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<th>ALK &gt;4 copies</th>
<th>P-value</th>
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<td>No</td>
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<td>4</td>
</tr>
<tr>
<td>Yes</td>
<td>35</td>
<td>13</td>
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<table>
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<th>Visceral disease</th>
<th>ALK &gt;4 copies</th>
<th>P-value</th>
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<td>No</td>
<td>34</td>
<td>7</td>
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<tr>
<td>Yes</td>
<td>19</td>
<td>10</td>
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Discussion

In the present study, we interrogate whether the ALK genomic alterations are of potential clinical relevance in patients with UC. Our study shows that ALK amplification and copy number gain but not fusions and translocations occurs in UC but is not associated with poor outcome in our patients with already bad prognosis.

ALK gene is located in 2p23 and encodes a transmembrane tyrosine kinase receptor involved in the development of nervous system during embryogenesis [20,21]. ALK gene was first shown to have a role in cancer as part of the fusion gene nucleophosmin (NPM)-ALK in anaplastic large cell lymphomas [9,10]. Preclinical studies show that tumors with aberrant activation of ALK tyrosine kinase are oncogene addicted to ALK intracellular signaling, and inhibition of the kinase by specific ALK targeting drugs results in tumor growth arrest and cell death [25].

The best well studied genomic alteration is the translocation seen in NSCLC patients. The majority of ALK rearrangements come from an interstitial deletion and inversion in chromosome 2p resulting in EMLA-ALK fusion gene product [22–27]. Although translocation is the most commonly identified mechanism for ALK activation, amplification and mutation have also been shown to act as oncogenic events [28–30]. The role of amplification and of copy number gain, as well as the role of deletion found in tumors like RMS remains to be determined [27,31–34].

The finding that several tumor types have been identified that have ALK as an oncogenic driver regardless of their cell of origin has prompted the creation of the term “ALKomas” implying a “beyond organ” concept classification assuming consequently responses to ALK inhibitors such as crizotinib [10,35]. Based on that, exploration of this concept is worthwhile in UC even if the frequency happens to be low.

In our cohort, aCGH-A found only some minor focal events in 3/70 specimens harboring non-significant alterations in ALK gene locus region. Since copy number gain has been recently associated with poor prognosis in several tumors like RMS, RCC and colorectal cancer (CRC), FISH analysis to assess the impact of copy number variations of ALK in our cohort was performed. In our patients, polysomy was frequently found in 90% of the cases [15,34]. The biological relevance of such finding is uncertain but could reflect genomic instability. The OS for patients with (2F+3–4F) vs. >5F was found to be 12 and 16 months respectively, however did not reach statistical significance (Figure 1). Likewise, there were no differences between ALK gene copy number gains and clinical features in all the different subgroups (2F, 3–4F and ≥ 5F). A plausible explanation for this lack of a significant difference between these groups is that it could be related to the natural history and the aggressive phenotype of our analysis cohort (metastatic disease requiring chemotherapy) with other genetic abnormalities beyond ALK gene copy number having a greater functional role in oncogenesis. Similarly, arm level ALK gene copy number gain as observed in this analysis may be unrelated to the driver oncogenic events.

Generally, patients with ALK copy gain have not shown to have detectable ALK protein expression as assessed by IHC except for a recent publication by van Gaal and colleagues [27,29,34,36]. In our series, no patient with gene copy gain or amplification tested positive by IHC. This is similar to that observed in CRC where increased ALK gene copy number did not translate to increased ALK protein expression [37]. However, this is not the case for patients being categorized as FISH positive, where this positivity strongly correlates with IHC. Of note, in lung cancer, a positive ALK FISH test and ALK IHC have been proposed as screening tools to detect ALK alterations being considered sufficiently sensitive to indicate treatment with crizotinib [37]. Moreover, in NSCLC, abnormal FISH signal patterns have varied from a single split signal to more complex signal patterns, such as deletions of the green 5′ end of the ALK probe, gain of the split or 5′ALK signal or both. These variant ALK FISH signals usually, but not always, represent an ALK translocation and therefore the finding of a loss of the 5′ALK signal has been considered to be a presumptive evidence of an ALK gene rearrangement [37].

In our series, the patient with a FISH positive result had a variant signal pattern that did not correlate with ALK protein expression as assessed by IHC. The case was interpreted as having a deletion in the ALK region due to loss of the green 5′ end of the ALK signal, after excluding the possibility it could be related to alternative translocation partners [Kinesin family 5B (KIF5B) and TRK-fused gene (TFG)]. In our patient we did not test for the rearrangement of other fusion partners to ALK such as C2orf44, KIF5B, NPM1, VCL, TFG, RET, ROS, and VCL [38–43]. These genes have all been shown to be partners of ALK in lung cancer [44].

Finally, ALK Mutations have been described in 10.4% of neuroblastoma samples but not in other pediatric tumors like RMS, Ewing sarcoma, or DSRCT and only occasionally in other solid tumors like CRC [45,46]. In lung cancers, ALK mutations appear to develop during clinical treatment with crizotinib and their generation probably renders EMLA-ALK resistant not only to crizotinib but also to other ALK inhibitors [47]. In our series, no ALK P946L mutation was observed. In our study the limitations of the platform used limits our conclusions of the mutation analysis. The absence or very low percentage of activating mutation of ALK described in the majority of adult solid tumors tested support our analysis that these alterations are not relevant events in UC.
Unfortunately, the suspected deletion in the ALK region was not confirmed with the sequencing approach used. Discordantly, mapping read pairs suggesting deletions resolved into correctly mapping read pairs that were in agreement with the insert size of the library when a single mismatch between read and reference genome was tolerated. Thus, these pairs do not support deletions at the ALK locus. The average read coverage across the ALK region was 5× and if only a small proportion of cells contained a deletion, we would not have been able to detect it. Because we suspect the deletion was close to the centromere, we might have missed it and might not have been able to confirm it by next generation sequencing.

To summarize, the increasing evidence that ALK alterations are seen in tumors from different origins highlights the concept of stratifying tumors according to oncogenic genotypes as opposed to tissue type when considering treatment strategies. The finding of ALK rearrangement together with no activating mutation in UC suggests that these alterations might not be pathogenic events in UC. The utility of testing ALK inhibitors in UC is not supported by this data, although in the absence of effective alternative agents testing ALK inhibitors may still be warranted.

In conclusion, ALK genomic alterations are rare and probably without prognostic implications in UC. The potential for testing ALK inhibitors in patients with deletions and copy number changes UC merits further investigation in a larger expanded cohort of UCs, but might be restricted to the infrequent finding of a FISH positive patient.

Supporting Information

Methods S1 Supplementary Methods. (DOCX)

Acknowledgments

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Author Contributions

Conceived and designed the experiments: JB TC JR. Performed the experiments: SS SR MS SM BB AM SS HH DB PK TC JR. Analyzed the data: JB IC SM RO JB PK TC JR. Contributed reagents/materials/analysis tools: JB TC JR. Contributed to the writing of the manuscript: JB AF SS CM HH DB PK TC JR.

References


Table 4. Comparison of OS between ≥SF patients and 2F+3–4F.

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<th>N</th>
<th>Death</th>
<th>Median OS</th>
<th>Hazard ratio</th>
<th>P-value</th>
<th>Adjusted hazard ratio</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>≥SF</td>
<td>2F+3–4F</td>
<td>53</td>
<td>34</td>
<td>12</td>
<td>1.1 (0.55, 2.16)</td>
<td>0.80</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.36 (0.60, 2.72)</td>
<td>0.38</td>
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