Case Report

Next-generation sequencing to dynamically detect mechanisms of resistance to *ALK* inhibitors in *ALK*-positive NSCLC patients: a case report

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Abstract: Tyrosine kinase inhibitors (TKIs) of the anaplastic lymphoma kinase gene (*ALK*) have significantly improved the quality of life and survival of non-small cell lung cancer (NSCLC) patients whose tumors harbor an *ALK* translocation. However, most of these patients relapse within 2 to 3 years as the tumor acquires resistance mutations. Unlike beaming and digital PCR (dPCR), which only allow a few mutations to be analyzed, next-generation sequencing (NGS) approaches enable the simultaneous screening of multiple genetic alterations even when the frequencies of the variants are very low. We present the case of a 52-year-old man who was diagnosed with an *ALK*-positive NSCLC and was treated with crizotinib and, subsequently, ceritinib. The analysis of serial liquid biopsies by NGS detected two asynchronous mutations arising in the *ALK* locus during disease progression, namely p.Gly1269Ala (c.3806G>C) and p.Gly1202Arg (c.3604G>A), that conferred resistance to crizotinib and ceritinib, respectively. The resistance mutations were detected independently at different times, and could be imputed to different metastatic lesions, thereby highlighting the importance of heterogeneity in advance disease. Plasma levels of *ALK* resistance mutations correlated well with tumor responses assessed by CT scans and bone scintigraphy, demonstrating that non-invasive tumor molecular profiling by NGS allows the efficient dynamic monitoring of *ALK*-positive NSCLC patients, and outperforms dPCR and beaming because more somatic mutations can be tracked over the course of the treatment. In conclusion, this case report illustrates the usefulness NGS to guide therapeutic decisions in *ALK*-positive NSCLC patients based tumor molecular profile upon disease progression.

Keywords: Anaplastic lymphoma kinase (*ALK*); case report; liquid biopsy; next-generation sequencing (NGS); non-small cell lung cancer (NSCLC)

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Introduction

Approximately 3–7% of non-small cell lung cancer (NSCLC) patients have a genomic rearrangement of the anaplastic lymphoma kinase (*ALK*) gene (1), the most prevalent fusion partner being the echinoderm microtubule-associated protein-like 4 (*EML4-ALK*) (2). The development of molecular targeted therapies such as tyrosine kinase inhibitors (TKIs) of the *ALK* locus has dramatically changed the quality of life and prognosis of *ALK*-positive NSCLC patients, with significantly better survival than that provided by chemotherapy [median OS approximately 50 and 12 months, respectively (3)]. However, despite the efficacy of *ALK* inhibitors, patients ultimately relapse

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Table 1 Clinico-pathological characteristics of the patient

Sex: Male
Age at diagnosis: 52 years
Smoking status: Former (10 cigarettes/day)
Histology: Adenocarcinoma
Clinical stage: IV (cT2N3M1)
Number of ALK-TKI lines during the study: 2
First line of treatment: Crizotinib
Second line of treatment: Ceritinib
Exitus date: 28/12/2016

within 2 to 3 years while receiving therapy (4). In order to detect molecular resistance mechanisms, tumor re-biopsy is required, although this is seldom feasible in routine clinical practice. Conversely, the analysis of liquid biopsies, can potentially overcome the aforementioned limitation, being a promising approach for identifying resistance mechanisms arising during disease progression. Resistance mutations in the *ALK* locus have been reported to occur in around 20% of *EML4-ALK* NSCLC patients treated with *ALK*-TKIs (5). Unlike patients with NSCLC who harbor an *EGFR* mutation, where the mechanism of resistance to first-line TKI treatment is mainly due to the p.Thr790Met (c.2369C>T) mutation (6), multiple resistance mutations to *ALK* inhibitors have been described in the *ALK* locus (7,8). On the other hand, distinct mutations in the *ALK* locus confer different sensitivities on a variety of *ALK*-TKIs (9,10); for example, pre-clinical evidence suggests that the p.Ile1171Asn (c.3512T>A) mutation confers resistance to crizotinib and alectinib, but not to ceritinib (5). Furthermore, the therapeutic arsenal against *ALK*-positive tumors has increased in recent years, with several *ALK*-TKIs recently being approved by the FDA (4). In routine clinical practice, these treatments are prescribed empirically upon first disease progression, rather than being based on the tumor molecular profile.

Digital PCR (dPCR), although an adequate approach for detecting and quantifying somatic mutations using liquid biopsies, can only detect a few already-known mutations at a given time, which means the method is of limited use for monitoring *ALK*-positive NSCLC patients. On the other hand, monitoring tumor burden by tracking single somatic mutations could be limited by tumor heterogeneity, especially in advanced stages (11). This limitation can be overcome by using novel NGS approaches that allow for simultaneous low-frequency variant calling of multiple mutations (12). We present the following case report in accordance with the CARE Guideline (13).

Case presentation

A 52-year-old Spanish man, a former smoker (10 cigarettes/day), with no significant past medical and family history, who developed cough, dysphonia and significant weight loss, with no relevant findings on physical examination, was diagnosed in June 2014 as having a poorly differentiated lung adenocarcinoma stage IV (cT2N3M1c TNM 7ª ed. with bone metastasis) by a positron emission computed tomography (CT) scan and a pathological examination. Clinico-pathological characteristics of the patient are described in *Table 1*.

A mutational study of the tumor biopsy was not performed in the hospital where the patient was initially diagnosed due to lack of material available for molecular analysis. Plasma samples were obtained in the Medical Oncology Department of Hospital Puerta de Hierro-Majadahonda. The patient was a participant in a research study that aimed to evaluate the clinical utility of liquid biopsies in NSCLC patients. The study protocol was approved by the Hospital Puerta de Hierro Ethics Committee (internal code PIE14/00064). The patient signed the appropriate informed consent on 9 October 2015.

The patient was initially treated with four cycles of cisplatin (75 mg/m^2) plus pemetrexed (500 mg/m^2) chemotherapy in August 2014 and he continued maintenance therapy with pemetrexed (500 mg/m^2). Unfortunately, after 12 months of chemotherapy a CT scan revealed progressive disease (PD), manifesting as new vertebral lesions.

A second biopsy was performed using a Vysis LSI ALK Dual Color Break Apart FISH probe kit (Vysis, Downers Grove, IL), which revealed an *EML4-ALK* rearrangement. Based on this new result, the patient received 250 mg crizotinib daily from October to December 2015. In December 2015, despite good treatment tolerance, a CT scan showed a new blastic metastasis in the axial skeleton and a slight increase in the size of the lung lesion (*Figure 1*), and the patient was diagnosed as having a PD. A plasma sample was obtained at this time and sequenced on an Ion S5™ Sequencer (Thermo Fisher, Palo Alto, CA)

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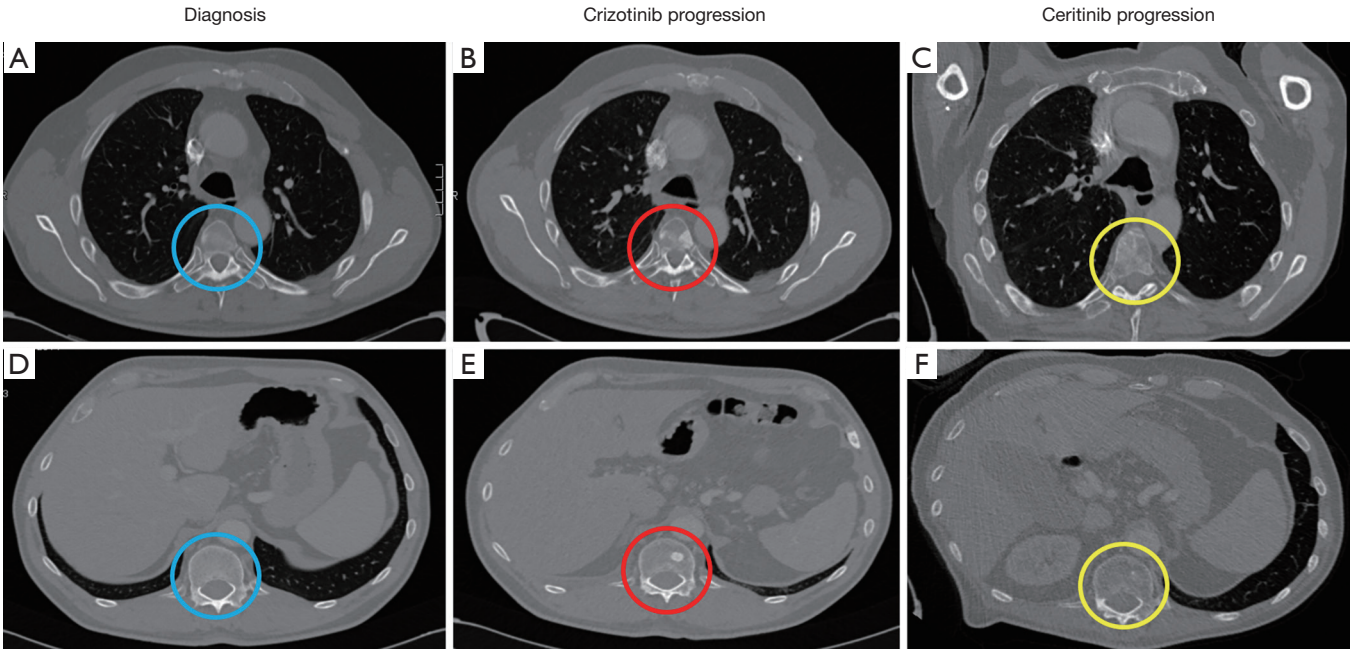

Figure 1 CT scans at times of diagnosis and progression during crizotinib and ceritinib treatment. (A) CT scan at time of diagnosis; (B) CT scan showing new T4 vertebral metastasis upon crizotinib progression; (C) The CT scan reveals a partial remission of T4 vertebral lesion with ceritinib treatment; (D) CT scan at time of diagnosis; (E) CT scan showing new T10 vertebral metastasis upon crizotinib progression; (F) The CT scan reveals a partial remission of T10 vertebral lesion with ceritinib treatment.

using the Oncomine™ Lung cfDNA Assay NGS panel (Thermo Fisher, Palo Alto, CA) to examine circulating tumor DNA (ctDNA). The NGS study revealed the presence of the p.Gly1269Ala (c.3806G>C) resistance mutation in the *ALK* gene (MAF =0.88%). This mutation was confirmed by dPCR (MAF =0.42%) using a custom TaqMan® assay in conjunction with a QuantStudio® 3D Digital PCR System (Applied Biosystems, South San Francisco, CA, USA). Next, using dPCR, we analyzed the p.Gly1269Ala (c.3806G>C) mutation in a plasma sample collected previously than the former. This technique did not detect the p.Gly1269Ala (c.3806G>C) mutation, correlating with tumor response to crizotinib at that time (November 2015) (*Figure 2*).

Upon progression to crizotinib treatment, the patient started treatment with ceritinib (600 mg per day). Throughout the course of this treatment there were no adverse events. Furthermore, ten plasma samples were collected and analyzed by dPCR. Partial response was assessed based on a CT scan performed in March 2016, correlating with undetectable plasma levels of p.Gly1269Ala (c.3806G>C) mutation, suggesting that ceritinib was able to eliminate the clone harboring the p.Gly1269Ala (c.3806G>C) mutation (*Figure 2*).

After 9 months of treatment (September 2016), the sequencing of a plasma sample using the Oncomine™ Pan-Cancer Cell-Free Assay NGS panel (Thermo Fisher, Palo Alto, CA) revealed the presence of the p.Gly1202Arg (c.3604G>A) resistance mutation in the *ALK* locus (MAF =1.28%) (*Figure 3A*), correlating with the symptomatology of the patient, who had reported a pain in his left hip. This mutation was confirmed by dPCR using another custom TaqMan® assay (MAF =2.12%) (*Figure 3B*).

Retrospective analysis of all 12 plasma samples collected by dPCR revealed that the p.Gly1202Arg (c.3604G>A) mutation was not present during the crizotinib treatment, but appeared between the fourth and sixth months (April-June 2016) after the start of the ceritinib treatment, albeit at very low allele frequencies (MAF =0.77% and 0.26%, respectively), coinciding with a hospital admission due to pneumonia. During the last 3 months of ceritinib therapy (October-December 2016), a dPCR study showed a significant increase of p.Gly1202Arg (c.3604G>A) mutation plasma levels (MAF = 2.11% in October 2016, and 6.70% in December 2016) (*Figure 2*), which was associated with the presence of the PD, as identified by scintigraphy performed

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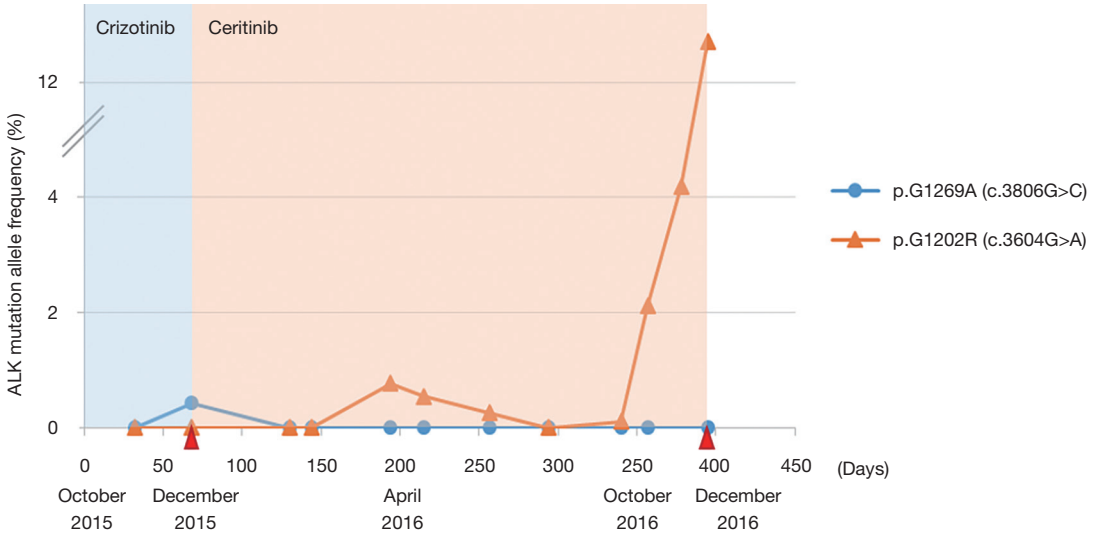

Figure 2 Monitoring molecular alterations using ctDNA in the two lines of *ALK*-TKI. The p.Gly1269Ala (c.3806G>C) mutation appeared upon crizotinib progression (December 2015) and was eliminated with ceritinib treatment. Conversely, the p.Gly1202Arg (c.3604G>A) mutation appeared during ceritinib treatment, increasing significantly upon ceritinib progression (October–December 2016). Red triangles indicate progressive disease.

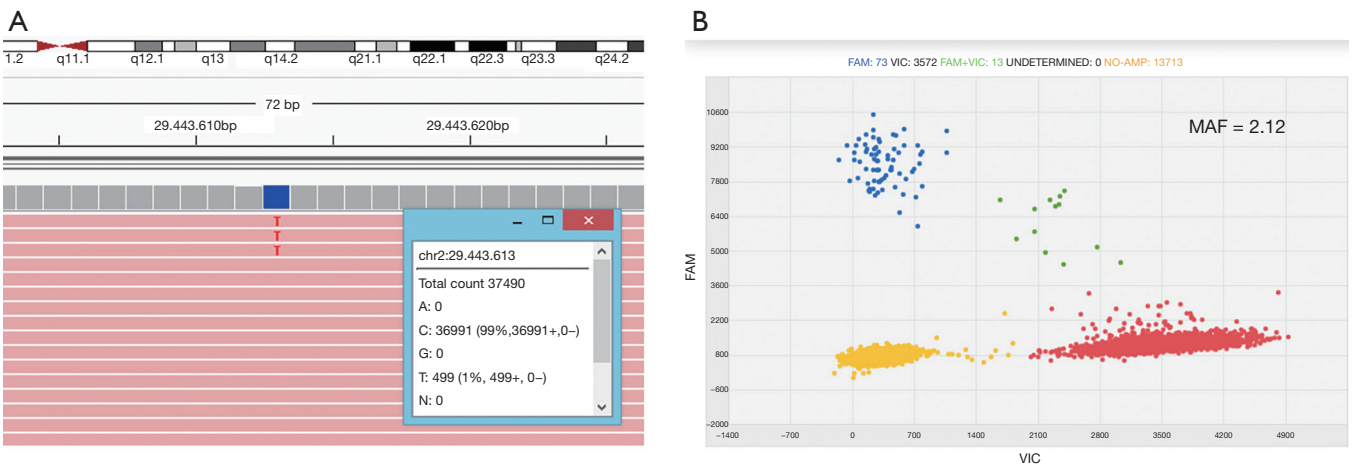

Figure 3 p.Gly1202Arg (c.3604G>A) mutation study. (A) p.Gly1202Arg (c.3604G>A) mutation detection by NGS: visualization of sequencing reads at the corresponding region by the Integrative Genomics Viewer; (B) On the dPCR scatter plot, the p.Gly1202Arg (c.3604G>A) mutation is labeled with FAM (blue data points), whereas the wild type is labeled with VIC (red data points). Green data points indicate the detection of both probes; yellow data points indicate no detection of probe.

in October 2016. Conversely, a CT scan on the same date showed a radiological response of some of the previous bone lesions detected at ceritinib initiation, and there was no evidence of new metastasis. In December 2016 the CT scan revealed PD with new bone blast lesions, especially in the femur and ischium (*Figure 4*). Finally, the patient died from the PD in December 2016, having attained an overall survival of 30 months.

Discussion

In this case report, we illustrate the usefulness of plasma

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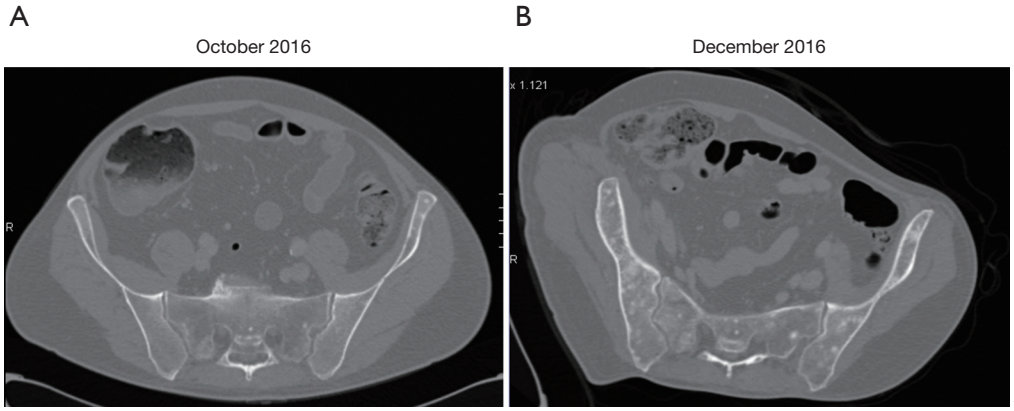

Figure 4 CT scans of new lesions observed upon ceritinib progression. (A) October 2016 CT scan with no evidence of PD; (B) December 2016 CT scan showed several new ischium lesions.

NGS profiling for detecting drug resistance mechanisms upon disease progression in NSCLC patients with an *EML4-ALK* rearrangement.

During the course of treatment, two *ALK* resistance mutations (p.Gly1269Ala (c.3806G>C) and p.Gly1202Arg (c.3604G>A)) were detected and quantified by NGS and dPCR. The p.Gly1269Ala (c.3806G>C) mutation has been described as a frequent event in crizotinib resistance in *ALK*-positive NSCLC patients, being detected in approximately 4% of cases (8). Next-generation *ALK*-TKIs such as ceritinib have demonstrated activity against the p.Gly1269Ala (c.3806G>C) mutation (14). In this way, plasma levels of this mutation dropped to undetectable levels soon after ceritinib treatment was initiated. Similarly, the p.Gly1202Arg (c.3604G>A) mutation occurs in 21% of patients following ceritinib treatment (5). Lorlatinib, the next-generation *ALK*-TKI, has demonstrated its efficacy against cells harboring the p.Gly1202Arg (c.3604G>A) mutation (15,16). Of note, the p.Gly1202Arg (c.3604G>A) resistance mutation was detected before disease progression, as ascertained by CT scan. In the same way, it has been reported that the p.Thr790Met (c.2369C>T) resistance mutation can be detected by ctDNA analysis approximately 50 days before PD is detected by CT scan in *EGFR*-positive NSCLC patients treated with a TKI (17,18), which suggests that ctDNA can complement imaging techniques to anticipate progression (19,20).

Technologies such as array-based dPCR or NGS allow the dynamic and quantitative analysis of somatic mutations in blood samples with high levels of sensitivity. However, dPCR can detect only a few, already-known genetic mutations in a single sample at a given time, while NGS enables the simultaneous screening of multiple mutations in multiple samples. Moreover, NGS allows to identify novel mutations. In this way the p.Gly1128Ala (c.3383G>C) mutation in *ALK* locus was recently identified in an *ALK*-positive NSCLC patient who progressed during treatment with crizotinib (21). As already mentioned, the use of dPCR is limited to monitoring a small number of mutations, and so is an unsuitable approach for screening resistance mutations in the *ALK* locus. However, as described in this case report, ctDNA profiling by NGS is feasible and can yield clinically useful information. Importantly, the laboratory turnaround time is short which make this a practical method for assisting oncologists in their clinical decision-making.

According to ctDNA analysis, in this case, ceritinib was able to eliminate the tumor cells that carried the resistance mutation p.Gly1269Ala (c.3806G>C), highlighting the high potency of the drug. Likewise, osimertinib, a third-generation *EGFR*-TKI, seems to be more potent than first- and second-generation TKIs such as erlotinib, gefitinib and afatinib, since it is more effective, in terms of survival, than standard *EGFR*-TKIs in first-line treatment (22). At the molecular level, unlike the first and second generations of *EGFR* inhibitors, in which the original *EGFR*-sensitizing mutation is always detected when disease progression occurs (23), osimertinib removes the tumor cells that carry both the original *EGFR* sensitizing mutation and the p.Thr790Met (c.2369C>T) resistance mutation in about half of the cases (24).

On the other hand, the two clones p.Gly1269Ala (c.3806G>C) and p.Gly1202Arg (c.3604G>A) were detected

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at different times during the course of disease, and could be imputed to different cancer lesions upon disease progression. In this way, the bone metastasis detected in the T4 and T10 vertebral lesions could be imputed to the clone harboring the p.Gly1269Ala (c.3806G>C) mutation, since these lesions were diagnosed at the same time as the mutation was detected in the blood. Moreover, both lesions showed partial remission with ceritinib treatment, in association with a decrease in the plasma levels of the p.Gly1269Ala (c.3806G>C) mutation (*Figures 1*,*2*). The ischium metastasis could be imputed to the clone harboring the p.Gly1202Arg (c.3604G>A) mutation (*Figure 4*). The CT scan clearly showed its occurrence to be associated with a significant increase in the plasma levels of this mutation, as assessed by dPCR (*Figure 2*).

In conclusion, molecular profiling of liquid biopsies using NGS is feasible and can be useful for monitoring tumor heterogeneity and clonal evolution during *ALK*-TKI treatment of NSCLC patients harboring *EML4-ALK* translocation, helping clinicians prescribe the most appropriate subsequent treatment lines, and improve the quality of life and outcome of their patients.

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Footnote

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at http://dx.doi.org/10.21037/tlcr.2020.02.07). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The patient participated in a research study that aimed to evaluate the clinical utility of liquid biopsies in NSCLC patients. The study protocol was approved by the Hospital Puerta de Hierro Ethics Committee (internal code PIE14/00064). Written informed consent was obtained from the patient for publication of this case report as well as the accompanying images.

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