Activating mTOR Mutations in a Patient with an Extraordinary Response on a Phase I Trial of Everolimus and Pazopanib

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Abstract

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Understanding the genetic mechanisms of sensitivity to targeted anticancer therapies may improve patient selection, response to therapy, and rational treatment designs. One approach to increase this understanding involves detailed studies of **exceptional responders**: rare patients with unexpected exquisite sensitivity or durable responses to therapy. We identified an exceptional responder in a phase I study of pazopanib and everolimus in advanced solid tumors. Whole exome sequencing of a patient with a 14-month complete response on this trial revealed two simultaneous mutations in mTOR, the target of everolimus. *In vitro* experiments demonstrate that both mutations are activating, suggesting a biological mechanism for exquisite sensitivity to everolimus in this patient. The use of precision (or “personalized”) medicine approaches to screen cancer patients for alterations in the mTOR pathway may help to identify subsets of patients who may benefit from targeted therapies directed against mTOR.

**INTRODUCTION**

Genetic characterization of sensitivity to targeted anticancer therapies has led to improvements in patient selection and treatment of multiple tumor types, including lung adenocarcinoma, colorectal adenocarcinoma, breast adenocarcinoma, and melanoma(1). Identification of patients with exquisite sensitivity and/or durable responses to targeted therapies – so called “**exceptional responders**” – may lead to improved patient selection and allow for more rational treatment designs.

Single-agent therapy for cancer only rarely results in durable disease control. At the same time, toxicities from therapeutic combinations often produce unacceptable toxicities(2). Therefore, careful pharmacokinetic investigation is needed during phase I studies to determine which combination therapies should be considered for further evaluation. VEGF receptor tyrosine kinase inhibitors (TKIs) often exhibit significant side effects, including rash, diarrhea, proteinuria, endocrine abnormalities, cardiotoxicity, and fatigue(3). Of these TKIs, pazopanib may be associated with less toxicity yet comparable efficacy(4–6). The lower toxicity and demonstrable activity of pazopanib make it an appealing candidate to combine with other targeted therapies. Inhibiting the PTEN/Akt/mTOR pathway by everolimus may complement VEGF receptor inhibition. Activation of mTOR, a downstream effector of Akt, has been shown in preclinical models to increase tumor cell proliferation and promote angiogenesis. Everolimus, an oral inhibitor of mTOR, decreases tumor and endothelial cell proliferation and tumor cell VEGF production, leading to antitumor and potential anti-angiogenic effects. Combining mTOR inhibitors with angiogenesis inhibitors in preclinical studies has been shown to augment both anti-angiogenic and antitumor effects(7, 8), suggesting that these two classes of agents might comprise an effective therapeutic combination.

Here, we describe the results of a phase I study of pazopanib and everolimus in advanced solid tumors. In this study, one patient with platinum- and taxane-refractory urothelial carcinoma experienced a complete radiologic response that lasted for 14 months. To elucidate the underlying mechanism of this extraordinary response, whole exome sequencing of tumor and normal DNA from this patient was performed.
RESULTS

Patients

Patient characteristics and the details of treatment are summarized in Table 1. Nine patients were enrolled in this trial (5 with urothelial carcinoma, 1 with small cell lung carcinoma (SCLC), 1 with non-small cell lung carcinoma (NSCLC), 1 with atypical carcinoid tumor of the lung, and 1 with adrenocortical carcinoma). All patients had experienced disease progression on standard therapies. The median number of cycles completed was 4 (range 1–13). Of the five patients with urothelial carcinoma, 1 patient received therapy for 13 cycles (28-day cycles; see Methods) and another for 6 cycles. Two patients completed 4 cycles, and one patient came off after the first cycle due to toxicity. The patient with NSCLC came off study after 2 cycles for toxicity and the patients with SCLC and atypical carcinoid tumor came off study for progression after 2 cycles. The patient with adrenocortical carcinoma completed 13 cycles of therapy and came off study per physician decision.

Safety

Two patients on pazopanib 600 mg daily and everolimus 5 mg daily experienced dose limiting toxicities (one grade 3 rash and pruritus and one grade 3 thrombocytopenia requiring a dose reduction) leading to dose de-escalation. Three patients were treated with pazopanib 400 mg daily and everolimus 5 mg daily. Since none of the first three patients experienced a protocol-defined dose-limiting toxicity, pazopanib 400 mg daily plus everolimus 5 mg daily, an additional 3 patients were treated at the MTD. Pazopanib 400 mg daily plus everolimus 5 mg daily was defined as the maximum tolerated dose (MTD).

Throughout the study, 5 of 9 treated patients experienced grade 3 or higher treatment-related toxicity (56%; 95% CI: 21.2%–86.3%). Hematologic toxicities during this trial included grade 3 febrile neutropenia and thrombocytopenia, each in 1 patient. Non-hematologic toxicities were varied, though many were expected based on the known toxicities of everolimus and pazopanib. One patient experienced a grade 3 pneumothorax, considered possibly related to study therapy. Other non-hematologic toxicities included anorexia (1 patient), bone pain (1 patient), diarrhea (2 patients; 1 patient with grade 3), fatigue (5 patients), dysgeusia (1 patient), headache (1 patient), hypertension (2 patients), mucositis (3 patients), musculoskeletal (muscle stiffness after prolonged periods of sitting, 1 patient), nausea (4 patients), palmo-planter erythrodysesthesias syndrome (2 patients), pneumonia (1 patient, grade 3), pruritus (1 patient, grade 3), rash (3 patients, 1 patients with grade 3), vomiting (3 patients), and weight loss (1 patient). Toxicities noted in laboratory evaluations were generally consistent with what is expected with both agents administered separately. Grade 3 and 4 laboratory toxicities included increased ALT, hypophosphatemia, and hypouricemia each in 1 patient, and increased lipase in 2 patients. All toxicities are summarized in Supplementary Tables 1 and 2.

Pharmacokinetics

Mean steady-state pharmacokinetic variables for everolimus in 7 patients and pazopanib in 6 patients, the MTD of the combination, are presented in Supplementary Table 3. Mean values of these parameters for the same dose and schedule of both drugs given alone in prior phase
I trials are shown in the table for comparison. The mean pharmacokinetic variables for pazopanib were in excellent agreement with data reported for a 400 mg daily dose given as a single agent(9). In contrast, the mean AUC\textsubscript{24\textsuperscript{ss}} of everolimus given concurrently with pazopanib was 41% greater than the mean value reported for a 5 mg daily dose given alone. These findings suggest that the pharmacokinetics of pazopanib are unaffected by everolimus whereas the apparent oral clearance of everolimus is markedly diminished by pazopanib, resulting in increased concentrations of the drug in whole blood, when the two agents were given together on a continuous basis.

**Antitumor activity**

One patient with metastatic urothelial carcinoma experienced a complete radiographic response that lasted for 14 months (Figure 1, A–D). Of the other urothelial carcinoma patients who were evaluable for responses, 3 had stable disease for 3.8 months, 5.6 months and 3.8 months respectively (Table 1). The patient with adrenocortical carcinoma also experienced prolonged stable disease for 13 months.

**Whole-exome sequencing**

To identify possible genetic mechanisms of sensitivity to everolimus/pazopanib in the patient with a complete radiographic response, we performed whole exome sequencing of the pre-treatment tumor and germline DNA from this patient. The DNA was sequenced to a mean depth of coverage of 74X (tumor) and 90X (normal), respectively. The coding regions from a total of ~25,000 genes were sequenced, with at least 80% of exons achieving 20X coverage or greater. 570 somatic single nucleotide variants (SNVs) were identified, of which 340 were missense, nonsense, or splice-site mutations (Supplementary Table 4). Of these 340 alterations, 5 were in genes in the Cancer Gene Census (CGC), a curated catalogue of genes for which mutations have been causally implicated in cancer: *PCMI, RANBP17, CTNNB1, FANCA*, and *TP53*. Two of the 340 SNVs are present in the Catalogue of Somatic Mutations in Cancer (COSMIC)(10), a database of somatic mutations found in human cancer: *CTTNB1* at an allelic fraction (AF) of 49% and *TP53* at an AF of 70%. There were 377 somatic small insertions or deletions (indels), of which 143 occurred in coding regions or at splice sites. Of these 143 indels, 8 were in CGC genes: *PDGFRA, ATRX, NUP214, CEBPA, MLL, MLL2, ARID1A, KDM6A (UTX)*. Indels in *PDGFRA, ATRX, NUP214*, and *CEBPA* were all present at an AF of < 10%.

Examination of the sequencing data for biologically plausible mechanisms of sensitivity to everolimus or pazopanib revealed two mutations in mTOR, the target of everolimus (Figure 2). Although 96 somatic nonsynonymous mutations in mTOR have been reported in the COSMIC database to date, neither mutation identified in this patient has been previously reported in human cancer. One mutation, mTOR\textsuperscript{E2419K} (AF 51%) (Figure 2A), is a well-described activating mutation in the kinase domain of mTOR, even though not identified in human cancer to date(11). The homologue of this mutation was identified in a fission yeast screen to identify single amino acid changes in yeast Tor2 (the fission yeast homologue of mTOR) that can cause hyperactivation of the pathway. There, a single mutation in Tor2, E2221K, was identified that conferred constitutive activation of TOR. The homologous mutation in human mTOR, E2419K, was subsequently generated in human cell lines and
shown to be constitutively activating through increased kinase activity and hyperactivation of the mTOR pathway. Notably, mTOR-mediated signaling remained sensitive to rapamycin in cells expressing mTOR\textsuperscript{E2419K} (11).

The second mutation we identified, mTOR\textsuperscript{E2014K} (AF 52%) (Figure 2B), occurs in the FKBP-rapamycin-binding (FRB) domain of mTOR. It has not been previously reported in clinical tumor samples, though it is present in the urothelial cancer cell line U-BLC1(12). In another study of potential hyperactivating mutations in mTOR, 2 FRB mutations, I2017T and A2020V, were shown to enhance kinase activity and cause hyperactivation of the mTOR pathway. Moreover, a mutant mTOR with both E2419K and I2017T exhibited higher activity of mTOR as compared to each individual mutation(13).

**Functional assessment of MTOR mutations**

To confirm that these mutations are activating, we assessed them using an established mTOR activity assay(11). Each mutant was individually overexpressed in HEK-293T cells and assayed for the degree of phosphorylation of S6K1, a downstream substrate of mTOR. As shown in Figure 2C, overexpression of mTOR\textsuperscript{E2419K} or mTOR\textsuperscript{E2014K} individually resulted in increased phosphorylation of S6K1 as compared to wildtype mTOR (Figure 2C, lanes 4 and 7 compared with lane 1). To determine if these mutations might exert additive activating effects, a variant of mTOR harboring both mutations was generated. Overexpression of this double mutant resulted in greater phosphorylation of S6K1 than did either single mutant (Figure 2C, lane 10 compared with lanes 4 and 7).

The mTOR residues E2014 and E2419 are conserved throughout all eukaryotic TOR genes. E2014 is located in a long helix that directly precedes the FRB domain (Figure 3, top magenta), termed the k\alpha1 helix(14). This helix is common to both mTOR and PI3 kinases and is important for the structural integrity of the kinase domain N-terminal lobe(14). E2419 (Figure 3, bottom magenta) is located near the negative regulatory domain, unresolved in the shown model. Though functional experiments show E2014K and E2419K to be activating mutations, neither make contact with the mTOR activation loop (Figure 3, light yellow) and thus are not likely to directly change its conformation. Similarly, neither mutation interfaces directly with bound rapamycin (Figure 3, cyan), and therefore would not be likely to directly alter binding to rapamycin or everolimus.

To test this, HEK-293T cells overexpressing wildtype or mutant mTOR were treated with rapamycin and the effect on S6K1 phosphorylation was assessed. As shown in Figure 2C, treatment with 0.1 μM rapamycin completely abrogated S6K1 phosphorylation by both wildtype and mutant mTOR, suggesting that these mutations remain highly sensitive to allosteric inhibition. In contrast, S6K1 phosphorylation was not affected by 1.0 μM pazopanib, though treatment with 10 μM pazopanib did diminish phosphorylation of S6K1 (Supplementary Figure 1).

Taken together, these results are consistent with the notion that the occurrence of two activating mTOR mutations within the same bladder tumor might contribute to an exquisite dependency on mTOR signaling and therefore an exceptional response to mTOR inhibition. Although it is possible that these two mutations occurred independently in different cells.
(each independently conferring sensitivity to a different subset of the tumor cells), the fact that both occurred with an AF of ~50% is highly suggestive that these mutations were heterozygous and were both present simultaneously throughout this tumor sample.

While we cannot rule out a direct contribution from pazopanib to the exquisite sensitivity in this patient, the doses of pazopanib required to overcome the effects of these mTOR mutations were more than 100-fold higher than those for rapamycin. Moreover, we were unable to identify any additional genomic alterations that might suggest a mechanism of pazopanib sensitivity. Although PDGFRA is a target of pazopanib, the indel identified in this gene (p.Y102fs) was detected at an AF of 2%, and is therefore unlikely to be functionally relevant in this tumor.

**DISCUSSION**

This phase I study of everolimus and pazopanib in patients with advanced solid tumors demonstrates that everolimus and pazopanib must be combined at doses 50% lower than their standard doses (pazopanib 400 mg and everolimus 5 mg daily). This is consistent with a pharmacokinetic drug interaction that decreases the apparent oral clearance of everolimus when combined with pazopanib, resulting in higher than expected concentrations of the drug in whole blood. The most common toxicities associated with therapy included fatigue, nausea, vomiting, diarrhea, and rash. In this study, several patients experienced prolonged stable disease. One patient with chemotherapy-refractory urothelial carcinoma demonstrated a complete radiographic response that lasted for more than one year. Only one other patient (with adrenocortical carcinoma) experienced a meaningful clinical benefit (stable disease for 13 months). Whole exome sequencing revealed the presence of 2 simultaneous activating mTOR mutations in the urothelial carcinoma tumor sample, suggesting a biological mechanism for exquisite sensitivity to everolimus in this patient.

In principle, tumors that exhibit a dependency on the mTOR pathway might be expected to show enhanced sensitivity to mTOR inhibition with drugs such as everolimus. Such dependencies could, conceivably, occur though any activating genomic alterations involving the mTOR pathway. In hamartoma syndromes such as tuberous sclerosis complex and Peutz-Jeghers Syndrome, inactivating mutations in the tumor suppressor genes TSC1, TSC2, and STK11 (LKB1) result in mTOR pathway activation and are targetable by TOR inhibitors(15–17). Similarly, a study describing 3 patients with malignant perivascular epithelioid cell tumors (PEComas) who had a significant clinical response to sirolimus demonstrated that two of these patients lacked expression of TSC2 and one patient had biallelic deletion of TSC1, suggesting that TSC1/2 loss may predict sensitivity to mTOR inhibition in PEComas(18). In a recent phase II study of everolimus in chemotherapy refractory urothelial carcinoma, whole genome sequencing of a single patient who had a durable complete remission was found to have somatic TSC1 mutation (along with a somatic NF2 mutation)(19). In addition, four of five additional patients with TSC1 mutations experienced tumor shrinkage with everolimus on that trial. However, beyond loss of TSC1/2, the basis for sensitivity to mTOR inhibition in cancer remains incompletely understood.
Here, we describe the first activating mutations in mTOR found in a patient tumor that was exquisitely sensitive to mTOR inhibition. One of these constitutively activating mutations, mTOR\textsuperscript{E2419K}, had previously been evaluated in human tissue culture based on its homology to an activating mutation observed in yeast\textsuperscript{(11)}. Though well studied \textit{in vitro}, this mutation has not heretofore been identified in human tumors. Similarly, the second mutation, mTOR\textsuperscript{E2014K}, has not previously been described. Indeed, very few activating mTOR mutations in human tumors have been described\textsuperscript{(20)}. In the COSMIC database, there are 96 confirmed somatic nonsynonymous mutations in \textit{MTOR} from 106 tumor samples. Recurrent mutations include R2505P (N = 4), S2215Y (3), E1799K (2), T1977R (2), L1433S (2), C1483F (2), and L1460P (2). To date, 3 of these mTOR mutations found in human tumors have been shown to be activating \textit{in vitro}. L1460P was identified as an activating mutation along with E2419K via the aforementioned fission yeast screen\textsuperscript{(11)}. More recently, additional mutations present in the COSMIC database were tested, and S2215Y and R2505P were shown to be activating \textit{in vitro}. Notably, all 3 of these mutations remain sensitive to rapamycin exposure \textit{in vitro}, though clinical information regarding the patients from whom these mutations were isolated is not available.

An additional mTOR mutation, L2431P, was recently identified in a patient with metastatic renal cell carcinoma and shown to be activating \textit{in vitro}\textsuperscript{(21)}. This kinase domain mutation was present in the majority of the samples taken from the primary tumor but absent from the metastatic biopsies. After 6 weeks of treatment with everolimus, imaging did not reveal any change in the dimensions of the primary tumor; this primary tumor was then surgically removed. Although this tumor did not apparently respond to a relatively short exposure to everolimus, it is not clear if this mutation is sensitive to allosteric inhibition of mTOR, in contrast to the mutations described in this study. Moreover, if the mTOR mutation detected in the renal cell carcinoma was present at a low allelic fraction, a clinical response may not have been apparent. The case described here provides evidence that activating mTOR mutations can confer clinically significant sensitivity to mTOR inhibition. Further clinical studies are needed to determine the specific circumstances under which patients with mTOR-mutated tumors are most likely to respond to mTOR inhibition.

Multiple activating alterations in the mTOR pathway, including mTOR, TSC1/2, and STK11 (LKB1), have now been shown to confer sensitivity to clinical mTOR inhibition. Systematic functional analysis of mutations in mTOR, as well as other members of the mTOR pathway, may help to identify activating mutations \textit{a priori}, generating a catalogue of activating mTOR-pathway alterations that may predict sensitivity to mTOR inhibition. Furthermore, routine screening of cancer patients for these alterations may help to identify a subset of patients who may respond to targeted therapies against mTOR, including everolimus and other rapamycin analogues as well as direct mTOR kinase inhibitors now in clinical trials.

More generally, the study of extraordinary responses in patients with cancer has the potential to identify novel genomic and molecular mechanisms of sensitivity to many different anticancer therapies. The ultimate goal of such an approach is to use those mechanisms for prospective identification of patients who might similarly respond to those specific therapies. Though promising, this approach also several challenges for precision
medicine going forwards. First, for each new plausible mechanism of extraordinary response identified, it may become important to characterize not only the functional significance of such alterations, but also whether the tumors in which they occur are truly dependent on the alterations. Because it is often difficult to recreate a dependency (and therefore sensitivity to a particular agent) in experimental models, the generation of patient-derived cell lines or patient-derived xenografts may eventually enable more definitive assessments of dependencies linked to specific genomic alterations in patients with the potential to be exceptional responders to anticancer agents.

It has become increasingly clear that the spectrum of actionable cancer gene alterations exhibits a “long tail” pattern, in which the vast majority of cancer genes are mutated at frequencies of <5% within any given histologic tumor subtype(1, 22). Since many such cancer genes and mutations remain undiscovered, the implication is that a large number of mutations in putatively actionable genes identified through tumor mutation profiling for precision medicine will never have been observed before. In the future, therefore, greater flexibility will be needed in the design of genomically-driven clinical trials to allow for a broad range of pathway alterations and to enroll small numbers of patients with less common mutations. Such innovations should enable more robust testing of many hypotheses generated by studies of exceptional responders and more rapid discovery of features that predict major clinical responses to anticancer agents.

**METHODS**

**Phase I clinical trial**

Patients ≥ 18 years old with metastatic or unresectable solid tumors for which standard curative or palliative measures do not exist, are not tolerable, or are no longer effective were eligible to enroll in this study. Detailed eligibility criteria can be found in the Data Supplement. This study was conducted with institutional review board approval in accordance with the Declaration of Helsinki and Good Clinical Practice and was registered with the National Institutes of Health (NCT01184326). Each patient provided written informed consent.

**Study design**

The primary endpoint of the study was to determine the maximum tolerated dose (MTD) and dose limiting toxicities (DLT) of combination therapy with everolimus and pazopanib. A standard 3 + 3 design was used starting with pazopanib 600 mg and everolimus 5 mg given orally once daily on a continuous basis during a 28-day cycle. The dose escalation was planned to alternate sequential escalations up to pazopanib 800 mg daily and everolimus 10 mg daily, or de-escalation to pazopanib 400 mg daily and everolimus 5 mg daily. Safety evaluation methodology is detailed in the Data Supplement.

**Pharmacokinetics**

Pharmacokinetic sampling was performed on day 15 of the first cycle of therapy to insure that steady-state conditions for the repeated dosing schedule for both drugs had been achieved. Blood specimens were obtained before initiating treatment, immediately before
dosing on day 15, at 1, 2, 4 and 6 hours after dosing, and before dosing on the following day. Detailed pharmacokinetic methodology can be found in the Data Supplement.

**Efficacy**

Objective tumor response, progression-free survival, and duration of response were evaluated by Response Evaluation Criteria in Solid Tumors (RECIST) Committee v. 1.1(23).

**Whole Exome Sequencing**

Sequencing studies were approved by the Dana-Farber/Harvard Cancer Center Institutional Review Board, and written informed consent was obtained from the patient. Whole exome sequencing was performed on the normal blood and tumor sample as detailed in the Data Supplement. The mean depth of coverage from the blood and tumor sample were 90- and 76-fold, respectively. Sequencing data were analyzed using tools to identify somatic point mutations and small insertions/deletions (indels) using established algorithms (see Data Supplement). Detailed analyses of all sequencing results are available in the Data Supplement (Supplementary Table 4).

**Experimental Analysis**

Expression plasmids containing mTOR were obtained and site-directed mutagenesis was performed as detailed in the Data Supplement. Transfections, mTOR activity assay, and immunoblot studies were performed using standard protocols (see Data Supplement). Authentication of HEK-293 cells was not performed. Physical and biologic containment procedures for recombinant DNA followed institutional protocols in accordance with the National Institutes of Health Guidelines for Research Involving Recombinant DNA Molecules.

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

**Acknowledgments**

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**REFERENCES**


STATEMENT OF SIGNIFICANCE

The study of exceptional responders represents a promising approach to better understand the mechanisms that underlie sensitivity to targeted anticancer therapies. Here, we identify two activating mTOR mutations in a patient with exquisite sensitivity to everolimus and pazopanib, suggesting an approach to identifying patients who might benefit most from mTOR inhibitors.
Figure 1. A 70 year-old man with metastatic urothelial carcinoma with a 14-month complete response to everolimus and pazopanib

Prior to the start of treatment, axial (a) and coronal (b) CT images without intravenous contrast demonstrate retroperitoneal adenopathy in left paraaortic and retrocaval stations (white arrows). Follow-up non-contrast axial (c) and coronal (d) CT images obtained two months after the start of therapy demonstrate resolution of the pathologically enlarged retroperitoneal lymph nodes (yellow arrows).
Figure 2. Identification of two activating mTOR mutations in a patient with a complete response to everolimus and pazopanib

(a–b) Representative genome images from the Integrated Genome Viewer (IGV) for two alterations in MTOR found in a patient with urothelial cancer who had a complete response to everolimus and pazopanib. The number of reads for the reference allele and the variant allele are shown for each alteration. (c) Effect of overexpression of mutant mTOR on phosphorylation of the downstream target S6K1. Constructs expressing mTOR\(^{E2014K}\), mTOR\(^{E2419K}\), mTOR\(^{E2014K/E2419K}\), or wildtype mTOR were co-expressed in HEK-293T
cells with HA-GST-tagged S6K1. The levels of both endogenous and exogenously expressed phosphorylated and total S6K1 as well as Flag-tagged-mTOR and GAPDH are shown for HEK-293T cells expressing the \textit{MTOR} mutations after a 6-hour incubation at 0 μM, 0.1 μM, or 1.0 μM rapacyn as indicated.
Figure 3. Structural model of \( m\text{TOR}^{E2014K} \) and \( m\text{TOR}^{E2419K} \)
Cartoon representation of the \( m\text{TOR}^\Delta\text{N}-\text{mLST8}-\text{rapamycin}-\text{FKBP12} \) model with color-coded domains. mTor residues E2014 and E2419 are indicated in magenta, with side chains shown as spheres. The model was created by superposing the FRB domains of the FRB-rapamycin-FKBP12(24) and the \( m\text{TOR}^\Delta\text{N}-\text{mLST8} \) structures(14). mLST8 is a requisite component of mTOR complex 1 and 2.
### Table 1

Patient characteristics.

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SD: stable disease; NE: not evaluable; CR: complete response; PR: partial response; PD: progressive disease.

^Patient only had 1 disease assessment and was reported to have SD at 3.8 months.