Analysis of serum protein biomarkers, circulating tumor DNA, and dovitinib activity in patients with tyrosine kinase inhibitor-refractory gastrointestinal stromal tumors

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Background: An exploratory translational analysis was conducted as part of a phase II study of dovitinib to assess the relevance of soluble serum proteins and circulating tumor (ct) DNA (ctDNA) as biomarkers in patients with tyrosine kinase inhibitor (TKI)-refractory gastrointestinal stromal tumors (GISTs).

Patients and methods: Predose serum samples were collected from 30 patients on day 1 of cycle 1 and cycle 2. Serum levels of angiogenesis-related proteins were assessed by enzyme-linked immunosorbent assay, and Beads, emulsions, amplification, and magnetics (BEAMing) assays were carried out to detect mutations in serum ctDNA.

Results: Dovitinib increased vascular endothelial growth factor (VEGF)165 (1.26-fold, \( P = 0.006 \)), VEGF-A (1.27-fold, \( P = 0.004 \)), placental growth factor (6.0-fold, \( P = 0.002 \)), fibroblast growth factor 23 (1.45-fold, \( P = 0.02 \)), and interleukin 8 (1.75-fold, \( P = 0.04 \)) levels, and decreased soluble vascular endothelial growth factor receptor (sVEGFR)-2 levels (0.8-fold, \( P = 0.001 \)). The changes in sVEGFR-2 were significantly associated with metabolic response determined by positron emission tomography (\( P = 0.02 \)) and progression-free survival (PFS; \( P = 0.02 \)). Secondary kinase mutations were identified in the ctDNA of 11 patients (41%), and these patients all had mutations involving KIT exon 17. Patients with secondary KIT mutations had significantly worse overall survival {median, 5.5 months [95% confidence interval (CI) 3.8–7.2 months]} than those with no detectable secondary mutations [9.8 months (95% CI 9.6–10.0 months); hazard ratio = 2.7 (95% CI 1.0–7.3); \( P = 0.047 \)].

Conclusions: Changes in sVEGFR-2 levels were associated with dovitinib-mediated antitumor activity. Genotyping of serum ctDNA with BEAMing is useful for the identification of resistant mutations potentially associated with poor prognosis in patients with GISTs.

Key words: gastrointestinal stromal tumor, dovitinib, biomarker, BEAMing, circulating tumor DNA

introduction

The standard therapy for inoperable locally advanced and metastatic gastrointestinal stromal tumors (GISTs) consists of imatinib and sunitinib [1]. A number of novel agents have been investigated for activity against imatinib/sunitinib-refractory GISTs; however, most of these drugs have failed to demonstrate efficacy in randomized trials [1, 2]. Before the results of the phase III trial favoring regorafenib as a third-line therapy in GISTs were available [2], we carried out a phase II trial of dovitinib, a novel tyrosine kinase inhibitor (TKI) targeting c-KIT, fibroblast growth factor receptor (FGFR), vascular endothelial growth factor receptor (VEGFR), and platelet-derived growth factor receptor \( \beta \) (PDGFR\( \beta \)) for patients with TKI-refractory GISTs [3]. In that study, dovitinib exhibited modest anticancer activity and a tolerable safety profile.

Identifying biomarkers that define patient subgroups that do benefit from dovitinib may provide further insight into its mechanism of action against GISTs and help refine our approach to future clinical trials. Therefore, an exploratory translational analysis was carried out for patients enrolled in the previous phase II study of dovitinib. As the main mechanism of action of dovitinib is thought to be antiangiogenic, soluble serum protein markers involved in the VEGF and FGF pathways were examined.

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Dovitinib activity may also be related to tumor genotype. Most GISTs harbor activating mutations in KIT or PDGFRα, and previous studies have shown that these mutations are associated with clinical outcome with both imatinib and sunitinib. Clonal evolution of GISTs harboring secondary kinase mutations results in acquired resistance to TKIs. As secondary mutations develop polyclonally, inter- and/or intratumoral heterogeneity may exist in each patient, particularly in those with advanced disease and multiple treatment failures. Although genotyping of tumor tissue is the gold standard method for detection of mutations, its utility is relatively limited in a salvage setting [4], considering the risk of complications associated with the invasive biopsy procedure and the potential failure to fully assess the molecular heterogeneity of the tumor. We therefore carried out mutational analysis on circulating tumor (ct) DNA (ctDNA) in serum using a novel technique, BEAMing (Beads, Emulsions, Amplification, and Magnetics) [5].

Methods

Patients

All 30 patients with TKI-refractory GISTs enrolled in the prior phase II study [3] were included in this biomarker analysis. Patients received oral dovitinib (500 mg) once daily on a 5 days-on/2 days-off schedule with a 28-day cycle. The study was approved by the Institutional Review Board of Asan Medical Center, Seoul, Korea, and conducted in accordance with the Declaration of Helsinki and Good Clinical Practice. Informed consent for serum protein biomarker analysis was obtained before enrollment in the phase II study of dovitinib, and informed consent for the mutation analysis of ctDNA was additionally obtained for all patients.

Serum Protein Biomarkers

Predose serum samples were collected on day 1 of cycle 1 (n = 30) and cycle 2 (n = 28). Enzyme-linked immunosorbent assays (ELISA) were used to assess serum levels of each soluble candidate. Candidate protein biomarkers for angiogenesis included VEGF165 (Invitrogen, Carlsbad, CA), VEGF-A (Ebioscience, San Diego, CA), soluble VEGFR (sVEGFR)-1 and sVEGFR-2 (Ebioscience), placental growth factor (PIGF; R&D Systems, Minneapolis, MN), interleukin 8 (IL-8; R&D Systems), basic fibroblast growth factor (bFGF; Invitrogen), and FGF23 (Millipore, Bedford, MA). All biomarkers were measured in duplicate at each time point and the average value of the two measurements was used for analysis.

BEAMing of ctDNA

Free circulating DNA was isolated from baseline serum samples using the QIAamp DNA purification kit (Qiagen). BEAMing assays were carried out on serum ctDNA samples for the detection of 29 KIT mutations (five in exon 11, one in exon 9, two in exon 14, two in exon 17, and one in exon 18), five PDGFRα mutations, and one BRAF mutation (supplementary Table S1, available at Annals of Oncology online). The details of the BEAMing methodology are described elsewhere [6]. BEAMing assays were conducted by Inostics GmbH (Hamburg, Germany).

Statistical Analysis

For each serum biomarker, the fold change from baseline to the end of the first cycle of dovitinib was calculated. The χ^2 test and Mann–Whitney U-test were used to assess the associations for categorical and continuous variables, respectively. The probability of survival was estimated using the Kaplan–Meier method and compared by log-rank test. The Cox proportional hazard model was used to estimate hazard ratios (HRs). The 24-week disease control rate (DCR) was defined as partial response (PR) or stable disease (SD) after 24 weeks of treatment. Progression-free survival (PFS) was defined as the time from the date of first dose to the date of disease progression or death. Overall survival (OS) was defined from the date of first dose to the date of death. P values of <0.05 were considered statistically significant, and all analyses were carried out using SPSS 18.0 (SPSS, Inc., Chicago, IL).

Results

Details of the clinical outcomes obtained with dovitinib were previously presented elsewhere [3], and are summarized in the appendix. Historical mutational data from archival tumor tissues collected before entry into this trial were available for 28 patients; KIT exon 11, KIT exon 9, and PDGFRα exon 18 mutations were found in 20 (71%), 5 patients (18%), and 1 patient (4%), respectively.

Serum Protein Biomarkers

Serum concentrations of all candidate proteins other than sVEGFR-1 and bFGF were detectable. Mean (standard deviation) baseline levels of VEGF165, VEGF-A, sVEGFR-2, PIGF, FGF23, and IL-8 were 431.2 (363.7), 849.6 (596.2), 5731.5 (1584.0), 27.1 (28.1), 19.0 (41.8), and 80.3 (96.1) pg/ml, respectively. Significant changes (P < 0.05 versus baseline) in these biomarkers were observed by the end of the first cycle of dovitinib (supplementary Figure S1, available at Annals of Oncology online). Dovitinib increased the levels of VEGF165 (1.26-fold, P = 0.006), VEGF-A (1.27-fold, P = 0.004), PIGF (6.0-fold, P = 0.002), FGF23 (1.45-fold, P = 0.02), and IL-8 (1.75-fold, P = 0.04), whereas sVEGFR-2 levels decreased 0.8-fold from baseline (P = 0.001).

For all six detectable proteins, baseline serum levels did not differ according to either metabolic response or 24-week DCR (P > 0.1 for all comparisons). However, a significantly larger decrease in sVEGFR-2 was observed in patients with metabolic PR than in those with metabolic SD or progressive disease (mean fold change, 0.63 versus 0.88; P = 0.02; Figure 1A), although the change in sVEGFR-2 levels was not associated with 24-week DCR (P = 0.39). Changes in the other five serum proteins did not correlate with either metabolic response or 24-week DCR (P > 0.1 for all comparisons).

To examine correlations between serum biomarkers and PFS, patient groups were categorized according to their baseline levels and fold changes. Notably, the fold change in sVEGFR-2 concentration was significantly associated with PFS (Figure 1B). Patients with the greatest decrease in sVEGFR-2 levels [quartile [Q1–Q3; median, 4.2 months [95% confidence interval (CI) 2.9–5.5 months]] following dovitinib treatment showed better PFS than those with smaller changes in sVEGFR-2 levels [Q4: 2.7 months [95% CI 0.4–5.0 months], HR = 0.4 (95% CI 0.1–0.9); P = 0.02]. Except for the change in sVEGFR-2, no correlation between serum biomarkers and PFS was observed.

Genotyping of ctDNA

BEAMing assays were carried out to genotype the serum ctDNA of all 30 patients. Although KIT exon 17 mutations were not...
analyzed for three patients due to insuffi- cient serum sample volumes, all other mutations were assessed for all patients. The three patients without data for KIT exon 17 mutations in ctDNA were not included in the analysis for secondary mutations. BEAMing of serum ctDNA identified primary kinase mutations in five (17%) patients (two for KIT exon 11 and three for exon 9). These were 100% concordant with the results of mutation analyses from tumor tissue taken from the corresponding patients.

Secondary kinase mutations were identified in 11 (41%) patients. Each of these patients had a mutation involving KIT exon 17. Three (27%) patients with KIT exon 11 mutations had more than one secondary KIT mutation (supplementary Table S2, available at Annals of Oncology online). Except for patient age, none of the baseline patient characteristics examined was significantly associated with the presence of secondary KIT mutations (Table 1).

The results of correlation analyses between the secondary KIT mutations detected in serum ctDNA and the activity of dovitinib are summarized in Table 2. Response rate, 24-week DCR, and metabolic response rate did not correlate with the presence of secondary serum ctDNA mutations. However, all four patients who achieved disease control by 24 weeks (including one patient with a PR) lacked secondary mutations, whereas tumors progressed within 24 weeks in all patients with secondary mutations. Figure 2 shows the relationship between survival outcomes and secondary mutations in serum ctDNA. Patients with secondary mutations had numerically shorter PFS [median 3.4 months (95% CI 2.2–4.6 months)] than those with no detectable secondary mutations [3.6 months (95% CI 2.6–4.6 months), HR = 1.7 (95% CI 0.7–3.9)], although this difference did not reach statistical significance (P = 0.23). OS was significantly worse in patients with secondary mutations [median 5.5 months (95% CI 3.8–7.2 months)] than in those who did not have secondary mutations [9.8 months (95% CI 9.6–10.0 months), HR = 2.7 (95% CI 1.0–7.3); P = 0.047].

In patients with secondary KIT mutations in serum ctDNA, baseline serum FGF23 levels [≤median (n = 5) versus >median (n = 6)] were significantly associated with PFS [median, 1.8 months (95% CI 0.8–2.8 months) versus 3.6 months (95% CI 3.2–4.0); P = 0.02; supplementary Figure S2A, available at

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**Table 1.** Baseline characteristics of patients categorized according to the presence of secondary KIT mutations in ctDNA

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>No secondary KIT mutations (N = 16)</th>
<th>Secondary KIT mutations (N = 11)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex, male</td>
<td>10 (63%)</td>
<td>8 (73%)</td>
<td>0.69</td>
</tr>
<tr>
<td>Age (years), median (range)</td>
<td>54 (35–70)</td>
<td>63 (52–76)</td>
<td>0.01</td>
</tr>
<tr>
<td>ECOG performance status 0–1</td>
<td>14 (88%)</td>
<td>7 (64%)</td>
<td>0.19</td>
</tr>
<tr>
<td>Primary site</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stomach</td>
<td>4 (25%)</td>
<td>4 (36%)</td>
<td>0.70</td>
</tr>
<tr>
<td>Small bowel</td>
<td>10 (63%)</td>
<td>7 (64%)</td>
<td></td>
</tr>
<tr>
<td>Others</td>
<td>2 (12%)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Primary kinase mutation (n = 25)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>KIT exon 11</td>
<td>10 (63%)</td>
<td>7 (78%)</td>
<td>0.88</td>
</tr>
<tr>
<td>KIT exon 9</td>
<td>4 (25%)</td>
<td>1 (11%)</td>
<td></td>
</tr>
<tr>
<td>PDGFRα exon 18</td>
<td>1 (6%)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Wild type*</td>
<td>1 (6%)</td>
<td>1 (11%)</td>
<td></td>
</tr>
<tr>
<td>TTP with 400 mg/day imatinib, median (range)</td>
<td>14.1 months (3.0–67.7)</td>
<td>27.2 months (5.2–71.2)</td>
<td>0.12</td>
</tr>
<tr>
<td>TTP with sunitinib, median (range)</td>
<td>6.7 months (2.1–30.0)</td>
<td>11.3 months (2.2–41.6)</td>
<td>0.30</td>
</tr>
<tr>
<td>Prior third or more lines of TKIs</td>
<td>6 (38%)</td>
<td>6 (55%)</td>
<td>0.45</td>
</tr>
</tbody>
</table>

*No mutations in KIT exons 11, 9, 13, and 17 and PDGFRα exons 12 and 18.

ECOG, Eastern Cooperative Oncology Group; TTP, time-to-tumor progression.
Furthermore, the fold change in sVEGFR-2 levels from baseline \[≤\text{median} (n = 6), \text{versus} >\text{median} (n = 3)\] also correlated with PFS \[\text{median, 2.7 months (95\% CI 0.8–4.6 months) versus 3.5 months (95\% CI 2.6–4.4 months); } P = 0.003; \text{supplementary Figure S2B, available at Annals of Oncology online}\]. However, in patients with no secondary ctDNA mutations, no biomarkers of PFS were identified.

**Discussion**

Our results demonstrate that dovitinib has pharmacological effects on both the VEGFR and FGFR pathways. Although baseline levels of candidate serum biomarkers were not predictive, changes in sVEGFR-2 levels were associated with dovitinib-mediated antitumor activity. Genotyping of serum ctDNA using novel BEAMing technology is a viable method for identifying resistant mutations in GISTs.

Dovitinib modulates serum levels of circulating proteins associated with VEGF and FGFR signaling. Dovitinib treatment increased VEGF\(_{165}\), VEGF-A, PIGF, and IL-8 levels and decreased sVEGFR2 levels, suggesting that dovitinib inhibited VEGF signaling in our patients. Consistent with previous studies [7], dovitinib-mediated inhibition of FGFR signaling was associated with increased levels of FGF23. It has been suggested that increased FGF23 levels result from compensatory upregulation following FGFR1 inhibition [7]. This suggests that the soluble serum proteins tested in this study may be pharmacodynamic markers for dovitinib activity and may act as predictive biomarkers in patients treated with dovitinib.

The baseline levels of the serum protein biomarkers were not predictive of clinical response to dovitinib in these patients. However, changes in sVEGFR-2 levels were significantly associated with clinical outcome. There was a greater decrease in sVEGFR-2 levels in patients with a metabolic PR than those who had metabolic SD or progressive disease. Patients who experienced smaller decreases in sVEGFR-2 levels following dovitinib treatment had reduced PFS. Considering that the decrease in sVEGFR-2 levels reflects VEGFR inhibition, this result suggests that the magnitude of VEGFR inhibition may reflect the level of dovitinib activity. In line with this, previous studies on other anti-VEGFR TKIs also demonstrated that decreased sVEGFR-2 levels were associated with improved efficacy [8]. Therefore, changes in sVEGFR-2 concentration may have predictive value for patients treated with dovitinib.

The major mechanism of acquired resistance to imatinib is the development of secondary kinase mutations. These are found in 50\%–70\% of patients who experience disease progression [9]. Secondary mutations are also associated with clinical response to subsequent TKI therapy [10]. Therefore, identification of secondary KIT mutations in ctDNA

<table>
<thead>
<tr>
<th>Outcomes</th>
<th>No secondary mutation (N = 16)</th>
<th>Secondary mutation (N = 11)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>24-week disease control</td>
<td>4 (25%)</td>
<td>0</td>
<td>0.12</td>
</tr>
<tr>
<td>Objective response by RECIST v1.0</td>
<td>1 (6%)</td>
<td>0</td>
<td>1.00</td>
</tr>
<tr>
<td>Metabolic response by PET-CT</td>
<td>3 (19%)</td>
<td>1 (9%)</td>
<td>0.62</td>
</tr>
<tr>
<td>PFS, median (95% CI)</td>
<td>3.6 months (2.6–4.6)</td>
<td>3.4 months (2.2–4.6)</td>
<td>0.23</td>
</tr>
<tr>
<td>OS, median (95% CI)</td>
<td>9.8 months (9.6–10.0)</td>
<td>5.5 months (3.8–7.2)</td>
<td>0.047</td>
</tr>
</tbody>
</table>

RECIST, response evaluation criteria in solid tumors; PFS, progression-free survival; OS, overall survival; CI, confidence interval.
secondary mutations may help to guide more personalized therapy against GISTs. However, repeat biopsies and genotyping after progression are not widely accepted for the management of patients with TKI-resistant GISTs because tissue biopsies provide limited information on molecular heterogeneity, and approximately two thirds of these patients experience multiple mutations [11]. Moreover, the risk of procedure-related complications may outweigh any potential benefit of the additional biopsy, and tumor lesions may not always be accessible to biopsy. To overcome the limitations of tissue biopsy, the utility of ctDNA as a biomarker has been actively investigated [4]. With the introduction of highly sensitive techniques, including BEAMing, the clinical advantages of less invasive ‘liquid biopsies’ have become clear, particularly for advanced cancers characterized by molecular heterogeneity [4, 5].

Our results showed that primary and secondary kinase mutations could be detected by genotyping serum-derived ctDNA from patients with TKI-refractory GISTs. Primary kinase mutations were identified in 17% of patients. As the BEAMing assay used in this study was not designed to detect most primary mutations, particularly those located in exon 11, the detection rate of primary mutations was relatively low, but the results of the mutation analysis from serum-derived ctDNA were 100% concordant with those derived from archival tumor tissue samples. Consistent with our results, in a subset analysis of a GRID trial for regorafenib in TKI-refractory GISTs [12], BEAMing of plasma-derived ctDNA found primary mutations in 27% of analyzed cases, and the concordance rate between BEAMing of ctDNA and sequencing of tumor tissue was 84%.

Secondary KIT mutations were found in the serum-derived ctDNA of 41% (11 of 27) of patients; 27% (3 of 11) of these cases had more than one KIT mutation. All patients with secondary KIT mutations in ctDNA had mutations involving the activation loop domain (exons 17 or 18). One patient (9%) had mutations present in both the ATP/drug-binding pocket (exons 13 and 14) and the activation loop domain. Consistent with our results, in the GRID trial, secondary KIT mutations were detected in 47% of patients; of these, KIT mutations were detected in exon 17/18 and 13/14 in 64% and 25% of patients, respectively, and 12% had mutations present in both domains [12]. The results of these two studies showed that secondary mutations are readily detectable in ctDNA by BEAMing and the majority of these mutations are located in the activation loop.

The presence of secondary KIT mutations in serum-derived ctDNA was significantly associated with reduced OS. This finding is supported by the results of a recent preclinical study that showed that dovitinib had limited activity against imatinib-refractory GIST cell lines harboring secondary KIT mutations (in exon 17 and 13) [13]. These results indicate that the detection of secondary mutations in ctDNA may be a promising tool for predicting drug efficacy and prognosis in patients with TKI-resistant GISTs. This also may be a useful enrichment strategy for future clinical trials of novel agents specifically targeting secondary mutations. Furthermore, baseline FGF23 levels and changes in sVEGFR-2 levels were associated with PFS in patients with secondary mutations. However, there was no correlation between the serum biomarkers examined and PFS in those patients where secondary mutations were not detected. This should be interpreted with caution, as only a small number of patients were included in the analysis. Nevertheless, our results suggest that dovitinib-mediated inhibition of the FGFR and VEGFR pathways may contribute to drug efficacy in patients with resistance mutations. However, the role of VEGFR and FGFR inhibition has not been established in the treatment of GISTs and remains to be defined in future studies.

Despite promising results using BEAMing for the detection of mutations in ctDNA, a major limitation of BEAMing is that it can only detect known mutations specifically included in the assay, and therefore cannot identify novel mutations or those not included in the assay. Optimal target mutation profiling for BEAMing of ctDNA and novel methodology, such as single-cell next-generation sequencing, are needed to overcome these limitations.

In conclusion, our analysis demonstrated that soluble serum proteins and secondary KIT mutations identified in serum-derived ctDNA are promising biomarkers for TKI-refractory GISTs. Further investigations of the clinical implications of soluble serum biomarkers and mutation analysis using serum-derived ctDNA are needed to validate these findings in a large patient population.

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disclosure
Y-KK has received honoraria and research grants from Novartis and Bayer, and is a consultant for both companies. Other authors have declared no conflicts of interest.

references
Survival follow-up and ipilimumab retreatment of patients with advanced melanoma who received ipilimumab in prior phase II studies


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Background: This report provides a survival update at a follow-up of >5 years (5.5–6 years) for patients with advanced melanoma who previously received ipilimumab in phase II clinical trials. Safety and efficacy data following ipilimumab retreatment are also reported.

Patients and methods: Patients who previously received ipilimumab 0.3, 3, or 10 mg/kg in one of six phase II trials (CA184-004, CA184-007, CA184-008, CA184-022, MDX010-06, and MDX010-15) were eligible to enroll in the companion study, CA184-025. Upon enrollment, patients initially received ipilimumab retreatment, extended maintenance therapy, or were followed for survival only. Overall survival (OS) rates were evaluated in patients from studies CA184-004, CA184-007, CA184-008, and CA184-022. Safety and best overall response during ipilimumab retreatment at 10 mg/kg were assessed in study CA184-025.

Results: Five-year OS rates for previously treated patients who received ipilimumab induction at 0.3, 3, or 10 mg/kg were 12.3%, 12.3%–16.5%, and 15.5%–28.4%, respectively. Five-year OS rates for treatment-naive patients who received ipilimumab induction at 3 or 10 mg/kg were 26.8% and 21.4%–49.5%, respectively. Little to no change in OS was observed from year 5 up to year 6. The objective response rate among retreated patients was 25%. Grade 3/4 immune-related adverse events occurred in 25%, 5.9%, and 13.2% of retreated patients who initially received ipilimumab 0.3, 3, and 10 mg/kg, with the most common being observed in the skin (4.2%, 2.9%, 3.8%) and gastrointestinal tract (12.5%, 2.9%, 3.8%), respectively.

Conclusions: At a follow-up of 5–6 years, ipilimumab continues to demonstrate durable, long-term survival in a proportion of patients with advanced melanoma. In some patients, ipilimumab retreatment can re-establish disease control with a safety profile that is comparable with that observed during ipilimumab induction. Further studies are needed to determine the contribution of ipilimumab retreatment to OS.

ClinicalTrials.gov: NCT00162123.

Key words: advanced melanoma, cytotoxic T-lymphocyte antigen-4, immunotherapy, ipilimumab, long-term survival, survival rate

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