Translational studies within the TAMRAD randomized GINECO trial: evidence for mTORC1 activation marker as a predictive factor for everolimus efficacy in advanced breast cancer


¹Department of Anatomopathology, Centre Léon Bérard, Lyon; ²Oncology Department, Gustave Roussy, Villejuif; ³Biostatistics and Therapeutic Evaluation Unit; ⁴Genomic Platform—Translational Research Laboratory, Centre Léon Bérard, Lyon; ⁵Medical Oncology Department, Centre Antoine Lacassagne, Nice; ⁶Medical Oncology Department, Centre Paul Papin, Angers; ⁷Oncology Department, Centre François Baclesse, Caen; ⁸Hematology and Oncology Department, Clinique de Valédour, Nice; ⁹Oncology Department, Centre Catherine de Sienne, Nantes; ¹⁰Oncology Department, Université Paris Descartes, AP-HP, Hôpitaux Universitaires Paris Centre, Site Hôtel-Dieu, Paris; ¹¹Hematology and Oncology Department, Centre Hospitalier Départemental Les Oudairies, La Roche-Sur-Yon; ¹²Department of Medicine, Institut Jean Godinot, Reims; ¹³Oncology Department, Centre d’Oncologie de Gentilly, Nancy; ¹⁴²B North Department, Department of Medical Oncology and Cancer Research Center of Lyon, Centre Léon Bérard, Lyon, France

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Background: Everolimus is an agent frequently associated with specific toxicities. Predictive markers of efficacy are needed to help define which patients could benefit from it. The goal of this exploratory study was to identify potential predictive biomarkers in the mammalian target of rapamycin (mTOR) complex 1 (mTORC1) activation pathway using primary tumor samples collected during the phase II tamoxifen plus everolimus (TAMRAD) trial.

Patients and methods: Tumor tissues were collected retrospectively from the TAMRAD trial. Immunohistochemistry was carried out using specific antibodies directed toward proteins that result in mTORC1 activation [canonical phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt)/mTOR or alternative pathways]. DNA was extracted from the tumor tissue; mutation screening in the PIK3CA gene (exons 9 and 20) and the KRAS gene (exons 2 and 3) was first carried out using Sanger direct sequencing, and then completed by next-generation sequencing for PIK3CA. An exploratory analysis of everolimus efficacy in terms of a time-to-progression (TTP) increase was carried out in each biomarker subgroup (high versus low expression referring to the median percentage of marked cells).

Results: A total of 55 primary tumor samples from the TAMRAD trial—25 from the tamoxifen-alone group and 30 from the tamoxifen/everolimus group—were evaluated for biomarkers. The subgroups most likely to have an improvement in TTP with tamoxifen/everolimus therapy, compared with tamoxifen alone, were patients with high p4EBP1, low 4EBP1, low liver kinase B1, low pAkt, and low PI3K. Among the 45 samples screened for mutation status, nine samples (20%; 95% CI 9.6 – 34.6) had a PIK3CA mutation. KRAS mutation was observed in one patient.

Conclusions: A positive correlation between late effectors of mTORC1 activation, a positive correlation between Akt-independent mTORC1 activation, and an inverse correlation between canonical PI3K/Akt/mTOR pathway and everolimus efficacy were observed in this exploratory analysis. However, these correlations need to be validated in larger studies before applying the findings to routine clinical practice.

Key words: breast cancer, biomarkers, everolimus, tamoxifen, mTORC1

introduction

Approximately 25% of patients with hormone receptor-positive (HR+) advanced breast cancer (BC) fail to respond to endocrine therapies because of de novo or acquired resistance [1]. The phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt)/mammalian target of rapamycin (mTOR) pathway has been shown to be one of the mechanisms mediating endocrine resistance [2, 3]. The mTOR inhibitor, everolimus, was recently studied in combination with endocrine therapy in the tamoxifen plus everolimus (TAMRAD) trial and in the Breast Cancer
TAMRAD trial. An exploratory analysis aimed to identify potential predictive biomarkers that inhibit signaling through mTORC1, this pathway is mediated through receptor tyrosine kinase, phosphatidylinositol-3,4,5-triphosphate (PIP3), and Akt. Phosphatase and tensin homolog (PTEN) is a specific negative regulator of this pathway because it dephosphorylates PIP3. Once phosphorylated, pAkt inhibits the tumor suppressor phosphatase and tensin homolog (PTEN) is a specific negative regulator of this pathway because it dephosphorylates PIP3. Once phosphorylated, pAkt inhibits the tumor suppressor protein, thus activating mTORC1 (supplementary Figure S1, available at Annals of Oncology online) [6, 7]. Hormone resistance models have shown this molecular pathway to be activated through receptor tyrosine kinase signaling and/or acquired mutation of PI3KCA; it is considered as one of the main mechanisms of mTORC1 activation and hormone resistance in estrogen receptor-positive (ER+) metastatic BC [3]. Nevertheless, other mTORC1 activation pathways are independent of Akt [7]. One of the most important Akt-independent pathways in cancer biology is the energy-sensing pathway regulated by AMP-activated protein kinase (AMPK) and its upstream kinase, the tumor suppressor gene liver kinase B1 [8, 9]. KRAS mutations have also been shown to activate the PI3K pathway independent of extracellular signaling [10]. Because everolimus is a novel targeted therapy that inhibits signaling through mTORC1, this exploratory analysis aimed to identify potential predictive biomarkers using primary tumor samples collected during the TAMRAD trial.

methods

The TAMRAD trial was a noncomparative, randomized, phase II study for patients with previous exposure to aromatase inhibitors. Patients were randomly assigned to receive either tamoxifen 20 mg/day plus everolimus at the recommended dose of 10 mg/day or tamoxifen 20 mg/day alone. The primary end point was a clinical benefit rate at 6 months. Secondary end points included time-to-progression (TTP), overall survival, and translational studies. Between March 2008 and May 2009, 111 patients were included in the trial (54% [49%] in the tamoxifen/everolimus arm and 57% [51%] in the tamoxifen-alone arm). The clinical benefit rate was 61% (95% confidence interval [CI] 47% to 74%) with tamoxifen/everolimus and 42% (95% CI 29% to 56%) with tamoxifen alone. TTP increased to 8.6 months with tamoxifen/everolimus from 4.5 months with tamoxifen (hazard ratio [HR] 0.54; 95% CI 0.36–0.81) [4].

The translational analysis reported here was preplanned, but tissue collection was not mandatory before patient inclusion in the study; hence, tissue samples were collected retrospectively after study completion in 2009. Initial tumor blocks were available from 55 patients. Because mTOR is the target of everolimus, immunohistochemistry (IHC) assessments of proteins involved in the canonical PI3K/Akt/mTOR pathway or alternative pathway (PI3Kp85, PTEN, LKB1, pAkt, eIF4E, 4EBP1, p4EBP1, S6RP, and pS6RP) were carried out [11, 12]. The hotspot mutational status of PIK3CA (exons 9–20) and KRAS (exon 2) was also assessed. Additional details on the IHC and mutational analysis are provided in supplementary Material, available at Annals of Oncology online.

exploratory analysis of everolimus efficacy within each biomarker subgroup

IHC biomarkers were dichotomized for the analysis (high versus low expression). We took into account only the percentage of marked cells (regardless of staining intensity) and set the cutoff value at the median percentages of cytoplasmic or nuclear marked cells. We used the median value as a cutoff because it allowed us to keep sufficient patients in each group, and we felt that the number was too small to select an optimum cutoff for each marker. Patients’ characteristics were studied according to high versus low expression of each biomarker. An exploratory analysis of everolimus efficacy in terms of TTP increase was carried out in each biomarker subgroup (high versus low expression). TTP was defined as the time from date of randomization to the date of first documented progression or death due to underlying cancer, and censored at the date of last tumor assessment for patients with no such event. In each biomarker subgroup, TTP of the tamoxifen/everolimus versus tamoxifen-alone group was estimated using the Kaplan–Meier method and compared using a log-rank test. Cox proportional hazard models with a term for treatment group were used (one model per biomarker subgroup) to estimate treatment HRs for progression between the two arms with 95% CIs.

results

patient clinical characteristics

Tissue samples for this exploratory translational study could be obtained from 25 patients in the tamoxifen group and 30 patients in the tamoxifen/everolimus group (supplementary Figure S2, available at Annals of Oncology online). Patient characteristics are described in Table 1. The median TTP was 10 months with tamoxifen/everolimus and 5 months with tamoxifen alone, translating to a 43% reduction in risk of progression (HR 0.57; 95% CI 0.32–1.00) with tamoxifen/everolimus. The HR observed for progression was consistent with the overall TAMRAD trial (HR 0.54; 95% CI 0.36–0.81).

analysis of driver mutations

A Sanger DNA sequencing analysis of samples obtained from 45 primary tumors identified a KRAS mutation (c.35G>A) in only one sample. Next generation sequencing (NGS) sequencing analysis of PIK3CA exons 9 and 20 revealed that the exon 9 mutation E542K in the helical domain of the catalytic subunit A was present in two samples. Exon 20 mutation H1047R, located in the kinase domain of subunit A, was present in seven samples. There was no correlation between PIK3CA mutational status and levels of expression of any of the biomarkers assessed by IHC. TTP of the nine patients with PIK3CA-mutated tumors was 7.5 months (95% CI 2.3–23.6) versus 6.8 months for the whole tumor population (n = 36; 95% CI 3.7–9.4).

IHC analysis

To identify any correlation that may exist between the expression of upstream and downstream markers in the mTOR activation pathway, an exploratory analysis was carried out to study the expression levels of pAkt and total LKB1 in patients with high baseline p4EBP1 expression, a marker of high mTORC1 activation. Positive and negative controls for IHC staining are
outlined in supplementary Figure S3, available at Annals of Oncology online. When evaluating the expression levels of cytoplasmic pAkt and total cytoplasmic LKB1 in patients with high baseline p4EBP1 expression, no clear correlation among pAkt, LKB1, and p4EBP1 could be determined.

**correlation between IHC results and PIK3CA mutation status**

No correlation was found when evaluating the relationships between the PIK3CA mutational status and protein expression of PI3K, cytoplasmic pAKT, or p4EBP1 in the corresponding IHC-assessed samples.

**predictive biomarker(s) of response to everolimus**

**predictive value of mutational analysis.** Of 45 patients, only 9 (20%; 95% CI 9.6–34.6) had a mutation of PIK3CA: 5 in the tamoxifen/everolimus arm and 4 in the tamoxifen-alone arm. These figures did not allow for any relevant statistical analysis.

**predictive value of IHC biomarker expression.** The ability of various proteins, as scored by IHC, to predict efficacy of the tamoxifen/everolimus combination was assessed in subgroups defined as having high or low expression of each biomarker (Figure 1). These results showed the potential predictive values of baseline expression levels of 4EBP1, p4EBP1, cytoplasmic LKB1, cytoplasmic pAkt, and PI3K. However, the efficacy of everolimus on TTP was found to be unrelated to the expression of pS6RP or eIF4E. Patients with low levels of 4EBP1 (n = 21) or high levels of p4EBP1 (n = 28) at baseline had a >60% reduction in risk of progression with the addition of everolimus (HR 0.39 [95% CI 0.14–1.08] and 0.35 [95% CI 0.15–0.86], respectively), whereas those with high 4EBP1 (n = 20) or low p4EBP1 (n = 19) seemed to benefit equally from both treatments (Figure 1). In patients with low cytoplasmic LKB1 levels (n = 22) at baseline, a 67% reduction in risk of progression was observed with tamoxifen/everolimus (HR 0.33; 95% CI 0.13–0.89), whereas those with high cytoplasmic LKB1 levels (n = 29) appeared to benefit less from everolimus. Patients with low cytoplasmic pAkt levels (n = 29) or low PI3K levels (n = 16) at baseline seemed to benefit more from tamoxifen/everolimus therapy than did patients with high cytoplasmic pAkt levels (n = 23) or high PI3K levels (n = 28) at baseline (Figure 1). The TTP survival curves of the selected biomarker subgroups are shown in Figure 2A–C.

**discussion**

The ultimate goal of managing BC has been to individualize therapy based on patient- and tumor-specific characteristics. Thus, it is of paramount importance to identify specific biomarkers that elucidate the presence or absence of aberrant signaling cascade in the tumor.

Recently, based on evidence that aberrant signaling through the PI3K/Akt/mTOR pathway may lead to endocrine resistance [2, 3], everolimus, an mTOR inhibitor, in combination with endocrine therapies was shown to reduce risk of progression in patients with HR+ human epidermal growth factor receptor2-negative BC compared with endocrine therapies alone [4, 5]. However, biomarkers that help in selecting patients who will benefit most from the PI3K/Akt/mTOR pathway inhibitors are still needed. The goal of this analysis, using tumor samples collected from the TAMRAD trial, was to identify potential predictive biomarkers that may prove useful in future trials and in routine clinical practice.

Of the 45 patients for whom we had enough tumor DNA to perform NGS, 9 (20%; 95% CI 9.6–34.6) had a PIK3CA mutation. This frequency is consistent with previous reports that 18%–47% of BCs harbor PIK3CA mutations [13, 14]. Nevertheless, the

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**Table 1. Baseline patient demographics and clinical characteristics**

<table>
<thead>
<tr>
<th></th>
<th>Population analyzed for the exploratory translational study (n = 55)</th>
<th>Overall population of the TAMRAD study (N = 111)</th>
</tr>
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<tbody>
<tr>
<td><strong>Disease stage</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bone only</td>
<td>9 (36)</td>
<td>30 (27)</td>
</tr>
<tr>
<td>Visceral</td>
<td>11 (44)</td>
<td>59 (53)</td>
</tr>
<tr>
<td>≥3 metastatic sites</td>
<td>4 (16)</td>
<td>29 (26)</td>
</tr>
<tr>
<td>Previous adjuvant TAM treatment</td>
<td>12 (48)</td>
<td>42 (38)</td>
</tr>
<tr>
<td>Previous chemotherapy</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adjuvant</td>
<td>18 (72)</td>
<td>57 (51)</td>
</tr>
<tr>
<td>Metastatic</td>
<td>4 (16)</td>
<td>28 (25)</td>
</tr>
<tr>
<td>Primary hormone resistance</td>
<td>12 (48)</td>
<td>54 (49)</td>
</tr>
<tr>
<td>Secondary hormone resistance</td>
<td>13 (52)</td>
<td>56 (51)</td>
</tr>
<tr>
<td>Median TTP, months (95% CI)</td>
<td>5.0 (2.7–9.3)</td>
<td>HR 0.57 (95% CI 0.32–1.00)</td>
</tr>
<tr>
<td></td>
<td>10.0 (6.0–17.4)</td>
<td>HR 0.54 (95% CI 0.36–0.81)</td>
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</table>

*Data are n (%), unless otherwise stated. CI, confidence interval; EVE, everolimus; HR, hazard ratio; TAM, tamoxifen; TTP, time to progression.
Because activation of mTORC1 is known to increase the level of expression of pS6K and p4EBP1 [15], it can be hypothesized that use of an mTOR inhibitor such as everolimus will improve outcomes in patients with higher basal expression of these downstream mTORC1 effectors. Consistent with this hypothesis, tamoxifen/everolimus seems to be more effective in improving TTP in patients with high baseline levels of p4EBP1 and low total 4EBP1 than in those with low p4EBP1 or high 4EBP1 (Figures 1 and 2A). Thus, 4EBP1 levels and p4EBP1 levels, if validated in larger trials, could be two potential biomarkers that can identify patients with activated mTORC1 who may then benefit from everolimus treatment.

mTORC1 has been shown to be activated through the canonical Akt-dependent PI3K/Akt pathway and through Akt-independent pathways [7, 16–18]. Several Akt-independent pathways, including the LKB1/AMPK and the Ras/mitogen-activated protein kinase kinase/extracellular-signal-regulated kinases pathways, have been shown to regulate mTORC1 through their regulation of tuberous sclerosis 2 [7, 17]. In this study, patients with low pAkt or low PI3K at baseline, suggesting minimal PI3K/Akt pathway activation, benefited from tamoxifen/everolimus therapy (Figures 1 and 2C). This appears counterintuitive, because signaling through the canonical PI3K/Akt/mTOR pathway has been shown to be highly prevalent in BC [19, 20]. Additionally, considerable focus has been given in recent years to use the mutational status of PI3K, such as PIK3CA mutation, as a predictive biomarker for mTOR activation and poor prognosis in BC [21]. However, studies have shown that PIK3CA mutation is associated with better survival compared with BC patients harboring wild-type PIK3CA [22, 23].

PIK3CA mutations have also been shown to increase sensitivity to tamoxifen and hormonal therapy [22, 24]. Recently, PIK3CA mutation was shown to be associated with a gene signature related with PI3K/Akt activation and also with relatively low mTORC1 activation [22]. Thus, the exact role of PI3K/Akt/mTORC1 activation in patients with ER+ BC is still debatable and may be quite different from the straightforward scheme derived from in vitro analysis [3]. Furthermore, Akt-independent mTORC1 activation can be associated with low levels of PI3K and pAkt, because it has been shown that mTORC1 activation leads to PI3K inhibition through a pS6-mediated negative feedback loop [25].

Consistent with the suggestion that patients with Akt-independent pathway-mediated mTORC1 activation benefit from everolimus therapy, patients with low-level baseline expression of LKB1, suggesting mTORC1 activation through Akt-independent pathways [7, 17], were found to have higher improvement in TTP (Figures 1 and 2B) with tamoxifen/everolimus therapy than did patients with high LKB1 expression. Thus, patients with mTORC1 activation through Akt-independent pathways at baseline may benefit from everolimus therapy; however, larger trials are needed to verify this observation.

This study has several limitations. First, it is a retrospective subgroup analysis of a randomized phase II trial, with a small number of patients on whom multiple markers were tested, so the statistical analysis is only exploratory. These results could be substantially different if more patients are evaluated. Secondly, the clinical validity of IHC evaluation of phosphorylated proteins in routinely processed human specimens has been questioned [26]. However, the intensity of staining also depends on the antibody performance. Consequently, when an antibody is robust for IHC, the impact of fixaion delay is minor [27]. Therefore, when available, we chose antibodies that had

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**Figure 1.** Forest plot of time to progression with tamoxifen/everolimus versus tamoxifen alone for each biomarker tested. EVE, everolimus; TAM, tamoxifen.

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>Hazard Ratio (95% CI)</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low p65RP expression</td>
<td>0.58 (0.24–1.41)</td>
<td>26</td>
</tr>
<tr>
<td>High p65RP expression</td>
<td>0.47 (0.19–1.12)</td>
<td>26</td>
</tr>
<tr>
<td>Low 4EBP-1 expression</td>
<td>0.39 (0.14–1.08)</td>
<td>21</td>
</tr>
<tr>
<td>High 4EBP-1 expression</td>
<td>0.82 (0.32–2.10)</td>
<td>20</td>
</tr>
<tr>
<td>Low p4EBP-1 expression</td>
<td>0.88 (0.34–2.30)</td>
<td>19</td>
</tr>
<tr>
<td>High p4EBP-1 expression</td>
<td>0.35 (0.15–0.86)</td>
<td>28</td>
</tr>
<tr>
<td>Low LKB1 expression</td>
<td>0.33 (0.13–0.89)</td>
<td>22</td>
</tr>
<tr>
<td>High LKB1 expression</td>
<td>0.67 (0.31–1.47)</td>
<td>29</td>
</tr>
<tr>
<td>Low pAkt expression</td>
<td>0.43 (0.19–0.96)</td>
<td>29</td>
</tr>
<tr>
<td>High pAkt expression</td>
<td>0.68 (0.28–1.68)</td>
<td>23</td>
</tr>
<tr>
<td>Low PI3K expression</td>
<td>0.15 (0.03–0.72)</td>
<td>16</td>
</tr>
<tr>
<td>High PI3K expression</td>
<td>1.09 (0.49–2.41)</td>
<td>28</td>
</tr>
<tr>
<td>Low Eif4E expression</td>
<td>0.74 (0.29–1.86)</td>
<td>22</td>
</tr>
<tr>
<td>High Eif4E expression</td>
<td>1.03 (0.35–3.04)</td>
<td>17</td>
</tr>
</tbody>
</table>
previously shown a wide range of staining across large series of tumors and had already been successfully used at our institution on samples from multiple sources [11, 28, 29]. Finally, all analyses were conducted on primary tumors, not metastatic tissue, so important molecular markers that evolve with metastatic disease may have been missed [30].

In conclusion, results from this exploratory analysis of protein expression assessed by IHC in primary tumors indicated that there may be a positive correlation between late effectors of mTORC1 activation and everolimus efficacy, a positive correlation between Akt-independent mTORC1 activation and everolimus efficacy, and an inverse correlation between canonical PI3K/Akt/mTOR pathway and everolimus efficacy. Owing to the small sample size and multiple retrospective analyses, however, these correlations are purely exploratory and need to be validated in larger studies.

Figure 2. TTP with tamoxifen/everolimus and tamoxifen alone. (A) Patients expressing high or low levels of p4EBP1. (B) Patients expressing high or low levels of LKB1. (C) Patients expressing high or low levels of PI3K. EVE, everolimus; TAM, tamoxifen; TTP, time to progression.
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disclosure

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references