mTOR inhibitors response and mTOR pathway in pancreatic neuroendocrine tumors

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Abstract

Medical therapy of pancreatic neuroendocrine tumors (P-NET) may take advantage of Everolimus treatment. However, the extent of therapeutic response cannot be predicted. This study was aimed to identify the possible predictive markers of response to Everolimus in P-NET. We found that Everolimus reduced the cell viability and induced apoptosis in primary cultures of 6 P-NET (P-NET-R), where the proliferative and antiapoptotic effects of IGF1 were blocked by Everolimus. On the contrary, 14 P-NET primary cultures (P-NET-NR) were resistant to Everolimus and IGF1, suggesting an involvement of PI3K/AKT/mTOR pathway in the mechanism of resistance. The response to Everolimus in vitro was associated with an active AKT/mTOR pathway and seemed to be associated with a greater clinical aggressiveness. In addition, a patient sensitive to Everolimus in vitro was sensitive to this drug in vivo also and showed a positive p-AKT immunohistochemistry (IHC) at tissue level. Similarly, a patient resistant to Everolimus treatment after surgery was not sensitive to the drug in vitro and had a negative p-AKT IHC staining. Therefore, present data confirm that P-NET primary cultures may be considered a model for testing medical treatment efficacy and that IHC characterization of p-AKT might help in identifying human P-NET who can benefit from Everolimus treatment. These data encourage conducting a prospective multicenter study involving different groups of P-NET patients treated with Everolimus.

Introduction

Pancreatic neuroendocrine tumors (P-NETs) are heterogeneous neoplasms originating from pancreatic neuroendocrine cells (Singh et al. 2015, Berardi et al. 2016), representing 1–4% of all pancreatic malignancies, with an increasing estimated annual incidence of 1 case/100,000 inhabitants (Al-Kurd et al. 2014). Based on the hormonal secretion, P-NETs are divided into functioning and nonfunctioning tumors, depending
on the clinical manifestation of a syndrome related to specific hormonal hypersecretion (Halfdanarson et al. 2008, Missiaglia et al. 2010, Ro et al. 2013). Radical surgery represents the first-line treatment for localized disease, whereas it is often not feasible for locally advanced or metastatic tumors (Vallea et al. 2014). The most effective pharmaceutical option in functioning tumors is represented by somatostatin analogues (SSAs) that are useful to achieve symptomatic control. These drugs have also been recommended for the control of P-NET growth by recent guidelines (Pavel et al. 2016).

The mammalian target of rapamycin (mTOR) is a serine threonine kinase located downstream the PI3K/AKT signaling pathway that plays a crucial role in mediating basic cellular functions (Jiang & Liu 2008). The PI3K/AKT/mTOR pathway is constitutively activated in different NETs, including those of pancreatic origin (Capdevila et al. 2011). The expression of mTOR pathway components has been suggested to correlate with clinical behavior and responsiveness to mTOR inhibitors (Righi et al. 2010, Qian et al. 2013, Zatelli et al. 2016). The mTOR inhibitor has recently shown antitumor activity in advanced, well-differentiated and moderately differentiated P-NETs (Yao et al. 2011), but resistance to this treatment may develop. However, the mechanisms regulating the sensitivity/resistance to mTOR inhibitors are still scantily elucidated, and predictive biomarkers of Everolimus efficacy in P-NET are still lacking. We recently demonstrated that responsiveness to Everolimus in human bronchial carcinoid primary cultures correlates with an active AKT/mTOR signaling pathway (Gagliliano et al. 2013). However, PI3K/AKT/mTOR signaling does not always predict the sensitivity to mTOR inhibitors and is difficult to assess (Zatelli et al. 2016). Therefore, the aim of this study was to investigate the possible predictors of sensitivity to Everolimus in P-NET, to identify patients who may benefit from treatment avoiding useless side effects in resistant patients.

Table 1

<table>
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<tr>
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<th>TNM</th>
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NA, not available; NEC, neuroendocrine carcinoma; NET, neuroendocrine tumor; NF, non-functioning.

*Poorly differentiated neuroendocrine carcinoma; *NET patients for whom 1 sample from the primary tissue and 1 sample from the lymph node metastasis were available, each generating a primary culture; **NET patients treated with Everolimus in vivo; ***NET patients for whom 2 primary tissue samples were available, each generating a primary culture.
in some cases, without lymph node (11/16) or distant metastases (13/16).

Tissue collection and primary cultures

Tissue samples were collected following the guidelines of the local committee on human research. Informed consent of the patients was obtained for disclosing clinical investigation and performing the in vitro study. Tissue samples were stored in RNA-later solution (Sigma) for expression studies; upon arrival in the lab, they were immediately frozen in liquid nitrogen under ribonuclease (RNase)-free conditions and stored at −80°C until protein isolation was performed. A portion of the fresh tissue was collected in culture medium for primary culture studies; upon arrival in the lab, the tissue was immediately processed for primary culture experiments as described previously, with minor modifications (Molè et al. 2011). Tumor cells were resuspended in F-12 with 10% fetal bovine serum (FBS) and antibiotics (Euroclone Ltd, Wetherby, UK), seeded at 2 × 10^4 cells/well in 96-well black plates and incubated at 37°C in a humidified atmosphere of 5% CO₂–95% air, as described previously (Zatelli et al. 2010a). After approximately 18 h, cells were treated with test compounds, with further evaluation of cell viability and/or caspase activity.

Compounds

Everolimus was provided by Novartis; IGF1 was purchased from PeproTek (Rocky Hill, NJ, USA). All other reagents were purchased from Sigma, if not otherwise indicated.

Viable cell number and caspase activation assessment

Cell viability and caspase activation were measured as described previously (Zatelli et al. 2006, Zatelli et al. 2010a,b) using the ATPlite kit (PerkinElmer Life Sciences) and the caspase-Glo 3/7 assay (Promega), respectively. Briefly, 20 P-NETs primary cultures, derived from 16 patients, were treated with or without 100 nM Everolimus for 48 h in the absence or in the presence of 100 nM IGF1. Luminous output (relative luminescence units, RLU) was recorded after 48 h for each assessment by the Envision Multilable Reader (PerkinElmer). Results are expressed as mean value ± S.E.M. percent RLU vs untreated control cells.

According to the response to Everolimus, samples were divided into primary cultures displaying a significant reduction (P < 0.05 vs untreated cells) in cell viability under Everolimus treatment, indicated as responders (P-NET-R), and into primary cultures in which Everolimus did not reduce cell viability, indicated as non-responders (P-NET-NR).

Kinase activity assay

Protein isolation from frozen human P-NET tissues was performed as described previously (Gagliano et al. 2013). RIPA buffer (Pierce) was used to dissolve tissues that were kept in ice for 30 min, and then centrifuged for 10 min. Protein concentration in the supernatant was measured by the BCA Protein Assay Reagent Kit (Pierce), as described previously (Tagliati et al. 2006). Each sample was evaluated in 3–6 replicates by assessing the same total protein amount. Phosphorylated levels of IGF1R (Tyr1135/1136), AKT (Ser473), mTOR (Ser2448) and 4EBP1 (Thr37/46) were measured using the AlphaScreen SureFire assays (PerkinElmer Life Sciences). Normalization against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) using the appropriate AlphaScreen SureFire assay kit was performed. Results are expressed as mean value ± S.E.M. AlphaScreen signal (counts).

Tissue microarray construction

Paraffin blocks were available for 15 P-NET patients. Tissue microarrays (TMA) were manually assembled from formalin-fixed, paraffin-embedded tissues. Three representative 1 mm diameter normal and tumoral tissue cores were taken from each block, for a total of 45 specimens. Multiple 3-μm sections were cut with a Leica microtome (Leica Microsystems), dried at 60°C and then transferred to adhesive-coated slides for immunohistochemical staining.

Immunohistochemistry

Immunohistochemistry (IHC) was performed by an anti-p-AKT (Ser473) antibody obtained by Abcam. Slides were de-paraffinized and re-hydrated in dewax solution (Leica Biosystems), and endogenous peroxidase activity was blocked with H₂O₂ solution for 4 min. After antigen retrieval using citrate buffer, pH 6.0 (15 min at 100°C), slides were incubated with the anti-p-AKT antibody (1:300) for 30 min, at room temperature. Immunoreactions were revealed by using the Bond Polymer Refine Kit on the automated system BOND RX (both from Leica Biosystems). Antigen-antibody complexes were detected using the cobalt-3,3’-diaminobenzidine (Co-DAB)
as the chromogen (Leica Biosystems). Samples were counterstained with hematoxylin and mounted in Aquatex (Merck). The specificity of all reactions was validated in parallel control sections omitting the primary antibodies for each IHC run. Endometrial cancers that are known to have, in the majority of cases, a PTEN mutation leading to PI3K/AKT pathway constitutive activation were used as positive controls.

**Evaluation of staining of tissue slides**

Tumor staining characteristics were reviewed and interpreted independently by two pathologists (A P and D N). IHC data were visually scored. Intensity and location (nuclear and membranous) were taken into consideration. The immunostaining intensity was evaluated subjectively as being negative (0), weak (1), moderate (2) and strong (3) and then the staining was classified into two categories: negative (no immunostaining) and positive (weak–moderate and strong immunostaining), in more than 10% of tumor cells.

**Statistical analysis**

Results are expressed as the mean ± S.E.M. Concerning the results of cell viability and caspase 3/7 activation experiments, a preliminary analysis was carried out to determine whether the datasets conformed to a normal distribution. The results were compared within each group and between the groups using ANOVA. If the F values were significant (P<0.05), Student’s paired or unpaired t-test was used to evaluate individual differences between the means. P values <0.05 were considered significant. For the AlphaScreen assays, after proof of homogeneity of variance and normality test, Student’s unpaired t-test was used. In case of non-normal distribution, Mann–Whitney test was used.

To compare the clinicopathological features, a preliminary analysis was carried out to determine whether the datasets conformed to a normal distribution and then Mann–Whitney test was used, due to not normal distribution for Ki-67, tumor size and number of mitoses. For sex, primary vs. metastatic tissue, pre-treatment with SSA, Octreoscan/68GA-PET uptake, site, TNM, stage, necrosis and tumor grade, chi-square test corrected for linearity was applied. Differences in age were assessed by the Student’s t-test. Data were analyzed using GraphPad (Prism v-5.0).

**Results**

**Effects of Everolimus and IGF1 on P-NET primary cultures**

Six P-NET primary cultures were defined as P-NET-R and 14 P-NETS were defined as P-NET-NR. As shown in Fig. 1A (upper panel), in P-NET-R, Everolimus significantly reduced the cell viability (−29.9%; P<0.05 vs untreated cells), an effect completely counteracted by co-incubation with IGF1. In P-NET-NR (Fig. 1B, upper panel), cell viability was significantly induced by Everolimus.
(+30.2%; *P* < 0.001 vs control cells) and was not affected by IGF1, alone or in combination with Everolimus.

To verify whether Everolimus modifies cell viability by influencing the apoptotic process, caspase 3/7 activation was evaluated. As shown in Fig. 1A (lower panel), in P-NET-R Everolimus significantly induced caspase activation (+73%; *P* < 0.001 vs untreated cells), an effect completely counteracted by co-incubation with IGF1. In P-NET-NR (Fig. 1B, lower panel), both Everolimus and IGF1 did not significantly affect caspase activation. These results indicate that P-NET may respond differently to Everolimus in vitro and that P-NET-NRs are not sensitive to IGF1 either, suggesting that IGF1 transduction pathway is not active in these tissues. Our data suggest that IGF1 pathway may be involved in the mechanism of resistance to Everolimus in vitro.

**IGF1/mTOR signaling pathway expression in P-NET tissues**

As IGF1 was capable of reversing the inhibitory effects of Everolimus on cell viability in P-NET-R, we investigated the levels of IGF1 downstream signaling proteins involved in mTOR pathway. Proteins isolated from frozen P-NET tissues, classified as P-NET-R and P-NET-NR on the basis of the results of primary cultures, were used to evaluate the expression levels of phosphorylated IGF1 R (p-IGF1 R), AKT (p-AKT), mTOR (p-mTOR) and 4EBP1 (p-4EBP1) by using AlphaScreen SureFire assay. As shown in Fig. 2A, we found that p-IGF1 R protein levels were significantly (*P* < 0.001) >2-fold lower in P-NET-NR compared with those in P-NET-R. Similarly, p-AKT levels were >2-fold lower in P-NET-NR compared with those in P-NET-R (Fig. 2B); however, statistical significance was not reached, possibly due to the small number of samples. Moreover, p-mTOR and p-4EBP1 protein levels were significantly (*P* < 0.02 and *P* < 0.001) >2-fold lower in P-NET-NR compared with P-NET-R (Fig. 2C and D). These data indicate that response to Everolimus in vitro is associated with an active AKT/mTOR pathway.

**Clinical and pathological correlations**

When the clinical characteristics of the patients were evaluated according to Everolimus responsiveness in vitro, there was no significant difference between patients with P-NET-R compared with those with P-NET-NR in terms of sex, age, primary vs metastatic tissue, pre-treatment with SSA, Octreoscan/68GA-PET uptake, site, TNM, stage, size, number of mitoses and necrosis. However, ki67 was significantly (*P* < 0.05) higher in P-NET-R (median 10%) than that in P-NET-NR (median 2%) (Fig. 3A). In addition, a trend for a linear correlation between tumor grade and response to Everolimus was found. All G3 P-NET responded to the treatment in vitro, whereas only 14% of G1 P-NET were P-NET-R (Fig. 3B).

**Expression of mTOR pathway components in P-NET and association with clinical outcomes**

To validate the identified putative markers of response to Everolimus in P-NET, the expression of p-AKT protein was
evaluated using IHC. Four tissue samples did not provide reliable results due to technical problems. Therefore, we obtained reliable results for 11 patients, 3 P-NET-R and 8 P-NET-NR. IHC for p-AKT was positive in all the paraffin-embedded tissues that corresponded \textit{in vitro} to 3 P-NET-R. IHC for p-AKT was positive in 5 out of 8 paraffin-embedded tissues that corresponded \textit{in vitro} to P-NET-NR. There was no statistically significant correlation between p-AKT positivity by IHC and responsiveness to Everolimus \textit{in vitro}, possibly due to the low number of available samples. The response to Everolimus \textit{in vitro}, as well as p-AKT protein levels in frozen tissues were also compared with the response to Everolimus \textit{in vivo}, in terms of tumor stabilization/progression and survival. Two patients had been treated with Everolimus after surgery due to the presence of non-resectable liver metastases. The first patient was a 56-year-old male operated on for a well-differentiated non-functioning G3 P-NET (T3N1M1, Ki67 = 25%), which turned out to be a P-NET-R \textit{in vitro}. The patient displayed stabilization of the disease during Everolimus treatment, with a progression-free survival of 21 months (still alive at the end of the study). Furthermore, he showed positive p-AKT IHC at tissue level (Fig. 4A). The second patient was a 65-year-old female operated on for a non-functioning G2 P-NET (T3N1M1, Ki67 = 20%), that turned out to be a P-NET-NR \textit{in vitro}. The patient displayed disease progression after 12 months of treatment with Everolimus, with a survival of 21 months (still alive at the end of the study). Data from IHC showed negative p-AKT staining (Fig. 4B).

**Discussion**

Our study shows that P-NET primary cultures are a useful model for testing medical therapy \textit{in vitro}. We also identified putative markers that may predict the response to Everolimus treatment \textit{in vivo}. In our settings, 30\% of human P-NET primary cultures responded to Everolimus treatment with a significant reduction in cell viability and induction of apoptosis, whereas the remaining 70\% did not. These data may seem in contrast with the demonstrated efficacy of Everolimus in prolonging PFS in advanced P-NET (Yao \textit{et al.} 2011, Wolin 2014). It should be underlined that we defined as responders those primary cultures displaying a significant cell viability reduction \textit{in vitro} after 48h. This parameter may not completely reflect the results obtained on clinical grounds in terms of PFS, which is influenced by many clinical variables and not by tumor growth only. On the other hand, our results may help in identifying those patients who may better benefit from treatment not only in terms of longer PFS but also in terms of tumor bulk reduction. Moreover, our series shows intra-patient and intra-tumor heterogeneity in primary cultures, further underlining P-NET heterogeneity. In addition, we show that the effect of Everolimus was influenced by IGF1 only in human P-NET tissues classified as responders to the drug \textit{in vitro}, but not in human P-NET tissues resistant to the drug. The importance of IGF1 in regulating NET proliferation is underlined by the evidence of an enhanced and constitutive expression of IGF1 and...
Everolimus responsiveness in P-NET

IGF1R in gastroenteropancreatic NET, which leads to autocrine and paracrine growth stimulation (von Wichert et al. 2000, Höpfnert et al. 2008). Moreover, we previously demonstrated that IGF1 exerts protective effects toward the anti-proliferative action of Everolimus in a group of medullary thyroid carcinoma primary cultures (Gentilin et al. 2015). Therefore, these data suggest that IGF1 pathway may play an important role in the development of Everolimus resistance. The latter represents a relevant issue because previous evidence demonstrated that some patients do not benefit from Everolimus treatment, due to the development of primary or secondary acquired resistance to this drug (Fazio 2015), despite a significant prolongation of PFS shown by the RADIANT-3 trial in a significant number of patients with P-NET (Yao et al. 2011). However, the precise mechanism underlying Everolimus resistance remains unknown (Zatelli et al. 2016).

Everolimus inhibits mTOR activity by interacting with the mTORC1 complex but not with mTORC2 complex. Selective blockade of mTORC1 by Everolimus treatment leads to an increase in mTORC2 activity and results in a positive feedback activation of AKT, by phosphorylation on Ser473, and of IGF1/IGF1R signaling due to inhibition of the S6K negative feedback (O’Reilly et al. 2006). As an evident association between the expression levels of PI3K/AKT/mTOR components and response to Everolimus in vitro was observed in bronchial carcinoids and in nonfunctioning pituitary adenomas (Zatelli et al. 2010a, Gagliano et al. 2013), we hypothesized that the investigation of mTOR pathway components could be useful to predict the responsiveness to Everolimus treatment in P-NET. In this setting, AlphaScreen data show that P-NET-R tissues display higher levels of p-IGF1R, p-AKT, p-mTOR and p-4EBP1 compared with those of P-NET-NR tissues, supporting the hypothesis that the expression of these proteins could be used to separate ‘responder’ tissues from ‘resistant’ tissues, in agreement with our previous results (Zatelli et al. 2010a, Gagliano et al. 2013). On this basis, our data suggest that the lack of response to Everolimus in vitro may be due to the low expression levels of PI3K/AKT/mTOR pathway components. Our results are in contrast with a recent study showing that high p-p70S6K protein levels are associated with a worse outcome under Everolimus treatment in NET (Benslama et al. 2016). However, the latter study was preformed including a very heterogeneous group of NET of different origin. Therefore, the results obtained by Benslama and coworkers may not reflect P-NET behavior (Benslama et al. 2016).

Besides putative molecular markers, we also investigated whether P-NET clinical characteristics may differ between responders and non-responders. We found a higher ki67 index in human P-NET-R compared with P-NET-NR and a trend for a linear correlation between tumor grade and response to Everolimus, suggesting that Everolimus responsiveness may associate with a greater clinical aggressiveness of P-NET.

Even though AlphaScreen represents a very sensitive method, validated by Western blot in bronchial carcinoids (Gagliano et al. 2013), it is not widely available. Therefore, we assessed IHC for p-AKT protein to validate the identified putative markers of response to Everolimus by means of a method that may be used also by other centers. The low number of available samples limited the significance of our findings as the correlation between p-AKT positivity by IHC and responsiveness to Everolimus in vitro did not reach statistical significance. The lack of correlation between AlphaScreen results obtained in fresh frozen tissues and IHC data for p-AKT expression is of importance. Although the fresh tissues were frozen after being stored several hours in RNAlater, which is a stabilizing RNA but does not influence protein phosphorylation, the paraffin-embedded tissues were processed routinely in the pathology lab with a variation in fixation times. The IHC protocols were performed on tissues that were fixed as large blocks and formalin is known to penetrate at 1 mm/h. During this time, phosphorylation levels of AKT may profoundly change (Baker et al. 2005), possibly explaining the different results obtained using these two different approaches. However, it is interesting to notice that p-AKT positivity by IHC was found in a P-NET-R patient who showed tumor stabilization under Everolimus treatment in vivo, suggesting that even P-NET-G3 may take advantage of this drug. On the contrary, IHC failed to demonstrate a strong p-AKT staining in a P-NET-NR who showed disease progression under Everolimus treatment in vivo. These findings suggest the presence of a good correlation between the responsiveness to Everolimus in vitro and that in vivo and provide the basis for further studies aimed at validating the identified putative markers of Everolimus responsiveness.

In conclusion, our results show that P-NET primary cultures may represent a suitable model for testing medical treatment efficacy. In addition, our data indicate that IGF1 pathway is involved in the development of resistance to Everolimus and that IHC characterization of p-AKT may help in identifying human P-NET who can benefit from Everolimus treatment. However, these preliminary data need to be confirmed and encourage conducting a prospective multicenter study involving different groups of P-NET patients treated with Everolimus.
Declaration of interest
M C Z has received consultant fees from Novartis and Genzyme. E d U has received consultant fees from Novartis and grant support from Sanofi. The other authors have nothing to disclose concerning a possible conflict of interest.

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Author contribution statement
S Falletta wrote the manuscript, performed primary cultures and AlphaScreen experiments and helped in setting up the immunohistochemistry experiments; S Partelli helped in providing the surgical specimens; C Rubini provided the paraffin-embedded tissues; D Nann performed the immunohistochemistry experiments; A Doria helped in providing the paraffin-embedded tissues; I Marioni provided support for the immunohistochemistry experiments and reviewed the manuscript; V Polenta helped in providing the clinical data; C Di Pasquale helped in performing the primary culture experiments; E degli Uberti provided support for the clinical correlations; A Perren supervised the surgical specimens; C Rubini provided the paraffin-embedded tissues; S Falletta wrote the manuscript, performed primary cultures and AlphaScreen experiments and helped in setting up the immunohistochemistry experiments; E degli Uberti helped in performing the primary culture experiments; C Di Pasquale supervised the surgical specimen provision and reviewed the manuscript and M C Zatelli wrote the manuscript, supervised the in vitro experiments and the project development.

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