mTOR expression and activity patterns in gastroenteropancreatic neuroendocrine tumours

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Abstract

Clinical trials indicate efficacy of drugs inhibiting the mammalian target of rapamycin (mTOR) in the treatment of gastroenteropancreatic neuroendocrine tumours (GEP-NET); however, information on detailed expression and activity patterns of mTOR in these tumours is sparse. We investigated the expression of mTOR and expression as well as phosphorylation of its downstream targets 4EBP1, S6K and eIF4E in a cohort of 99 human GEP-NET by immunohistochemistry. We correlated our findings with clinicopathological variables and patient prognosis. We found that 61, 93, 80, 69, 57 and 79% of GEP-NET were positive for mTOR, 4EBP1, cytoplasmic phospho-4EBP1 (p-4EBP1), nuclear p-4EBP1, phospho-S6K (p-S6K) and phospho-eIF4E (p-eIF4E) respectively. mTOR expression and activity were higher in foregut than in midgut tumours. In foregut tumours, expression of mTOR was higher when distant metastases were present \( (P=0.035) \). Strong mTOR activity was associated with higher proliferative capacity. In patients with stage IV midgut tumours, strong p-S6K expression was associated with poor disease-specific survival \( (P=0.048) \). In conclusion, mTOR shows considerable variations in expression and activity patterns in GEP-NET in dependence of tumour location and metastatic status. We hypothesise that these differences in mTOR expression and activity might possibly influence response to mTOR inhibitors.

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Introduction

With an incidence rate of about 2.5–5 cases per 100 000 person-years, gastroenteropancreatic neuroendocrine tumours (GEP-NET) are rather rare neoplasms when compared with adenocarcinomas of the same locations (Modlin et al. 2008). However, it is worth noting that the incidence of these tumours has risen tremendously over the last decades (Modlin et al. 2003, Yao et al. 2008a). Today, GEP-NET are treated in multidisciplinary approaches including surgery, biotherapy, chemotherapy as well as molecular targeted therapy (Plöckinger & Wiedenmann 2007, Oberg & Jelic 2008). Unfortunately, all improvements in the understanding and treatment of this disease have not resulted in significantly prolonged overall patient survival (Modlin et al. 2008), therefore novel treatment strategies for these tumours are still urgently needed.

Recently, the mammalian target of rapamycin (mTOR) inhibitors temsirolimus (Rini 2008) and everolimus (Sánchez-Fructuoso 2008b) have entered late-phase clinical trials in a broad variety of solid human tumours. Both substances have been tested for their activity in phase II studies (Duran et al. 2006, Yao et al. 2010) in a heterogeneous set of neuroendocrine neoplasms, and everolimus proved to be effective in the first place, especially in pancreatic...
neuroendocrine carcinomas. Tissue-based predictive biomarkers for response to everolimus are currently lacking. However, expression of mTOR pathway components has been suggested as a predictive biomarker for response to temsirolimus (Duran et al. 2006).

The mTOR protein is a central component of two protein complexes intimately involved in carcinogenesis (Sabatini 2006). mTOR complex 1 (mTORC1), also containing raptor and mLST8, phosphorylates the eukaryotic translation initiation factor 4E-binding protein (4EBP1) and the ribosomal S6 kinase (S6K1). Phosphorylation of 4EBP1 in turn leads to a dissociation of the protein from eIF4E, an important regulator of translation, subsequently eIF4E gets phosphorylated and activated (Whalen et al. 1996). Activation of these factors, finally, leads to enhanced cancer cell growth, prolonged cancer cell survival and neoangiogenesis (Hay & Sonenberg 1996). mTOR complex 2 (mTORC2), which does not contain raptor but rictor and mSin1, is less well understood (Sarbassov et al. 2005). However, there is evidence that this complex is able to activate AKT thereby inducing anti-apoptotic and pro-proliferative stimuli.

In this study, we aimed to investigate the expression and activity state of mTOR and its downstream targets 4EBP1, S6K and eIF4E in a large cohort of gastro-enteropancreatic neuroendocrine foregut and midgut tumours. We correlated our findings with clinicopathological variables and patient prognosis.

**Patients, materials and methods**

**Patient characteristics**

A total of 99 patients with GEP-NET of the foregut (47, 47.5%) and midgut (52, 52.5%), who received surgical treatment at the Charité University Hospital between 1983 and 2007, were included in the study. In detail, 9 (9.1%) tumours were gastric, 6 (6.1%) were duodenal, 31 (31.3%) were pancreatic, 3 (3%) were jejunal and 50 (50.5%) were ileal. In 70 cases, tissue from the primary lesion was available, 10 and 19 tissue specimens were from nodal and distant metastases respectively. In addition, in 33 cases, the primary tumours as well as nodal metastases were available for analysis. In 23 cases, the primary tumour and corresponding distant metastasis could be investigated. All cases were validated by immunohistochemistry in the routine diagnostic setting. By convention, antibodies against chromogranin A and synaptophysin were used to ensure neuroendocrine differentiation. If only one of the markers was positive, cluster of differentiation CD56 was stained in addition. Only cases with expression of two markers were designated as NETs. None of the patients included in this study had a hereditary syndrome, such as von Hippel–Lindau disease or multiple endocrine neoplasia nor were there familial cases without a known germline mutation. The mean age of patients with foregut tumours was 53.0 years at the time of the diagnosis. The mean age of patients with midgut tumours was 58.2 years. Of 99 patients, 51 (51.5%) were male. There was no association of sex distribution with tumour location in foregut or midgut. Follow-up data were available for almost all patients. However, since NET-related death occurred in the minority of patients with low-stage and low-grade tumours and since NETs of different locations are known to have significantly different survival (Plöckinger & Wiedenmann 2007), we decided to perform survival analysis exclusively in the homogenous group of midgut patients with stage IV disease. In this subgroup of 39 patients for whom data were available, 8 (20.5%) died of their disease after a mean follow-up time of 78.0 months. Those patients still alive at the endpoint of analysis were followed for a mean time of 48.3 months (range 3.5–210.7 months). We were able to gather treatment data in 21 of these 39 stage IV midgut patients. Of these, 11 received somatostatin receptor antagonists, 7 patients received no further treatment and 3 patients received other treatment regimens (including conventional chemotherapeutics). NETs were re-graded and re-staged according to the novel consensus proposals for GEP-NET and according to the WHO (7th edn; Rindi et al. 2006, 2007, Sobin et al. 2009). The clinicopathological characteristics of the patients are given in Tables 1 and 2. The study has been approved by the Charité University Ethics Committee (EA1/06/2004).

**Tissue**

For the evaluation of mTOR, 4EBP1, phospho–4EBP1 (p-4EBP1), phospho–S6K (p-S6K) and phospho–eIF4E (p-eIF4E) expression, tissue microarrays were generated using a precision instrument (Beecher...
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For one case, data on p-S6K and p-eIF4E expression were missing. Data on clinicopathological variables were missing for stage, T, N and M in a few cases in the respective subgroup analysis. For grade, only primary tumours were evaluated (n=33); in this subgroup, grading was not possible in two cases.

aχ² test for trends.
bFisher’s exact test.
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<th>Characteristics</th>
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For one case, data on mTOR, 4EBP1, p-S6K and p-eIF4E expression were missing. For some cases, data on clinicopathological variables were missing for stage, T, N and M in the respective subgroup analysis. For grade, only primary tumours were evaluated (n=37); in this subgroup, grading was not possible in one case.

a$^{2}$ test for trends.

bFisher’s exact test.
A representative tumour-bearing slide was selected for each case by a board certified pathologist with a special interest in GEP-NET pathology (WW). Typical tumour areas from the centre of the lesion as well as from the invasive margins were marked on the respective H&E slides. Subsequently, three tissue cylinders of 1.5 mm diameter were punched from each tumour-bearing donor block and transferred to a tissue microarray paraffin block. In addition, from every corresponding donor block, one conventional 2 μm paraffin section was cut for Ki-67 staining.

As normal reference control, ten cases of pancreatic tissue without significant pathology were investigated for the expression of the respective pathway components. Normal tissue was evaluated on conventional paraffin sections. Tissue was taken from patients with pancreatic NETs well away from the tumour.

**Immunohistochemistry**

Anti-mTOR antibody, anti-4EBP1, anti-4EBP1 phosphorylated at Thr70 (p-4EBP1), anti-eIF4E phosphorylated at Ser209 and anti-S6K phosphorylated at Thr389 antibodies were obtained from Cell Signaling Technology (Danvers, MA, USA). For immunohistochemistry, 3 μm paraffin sections were cut and incubated with anti-mTOR (1:50), anti-4EBP1 (1:50), anti-p-4EBP1 (1:25), anti-p-S6K (1:100) and anti-p-eIF4E (1:50) antibodies. The omission of the primary antibody served as negative control.

Ki-67 staining was performed in a Benchmark XT autostainer (Ventana, Tuscon, AZ, USA) according to the manufacturer’s protocol.

**Evaluation of staining of tissue slides**

Staining of mTOR, 4EBP1, p-4EBP1, p-S6K and p-eIF4E in tumour tissue was scored by applying a semi-quantitative immunoreactivity scoring (IRS) system, as described previously (Darb-Esfahani *et al*. 2009). Briefly, category A documented the intensity of staining as 0 (no immunostaining), 1 (weak), 2 (moderate) and 3 (strong). Category B documented the percentage of immunoreactive cells as 0 (none), 1 (<10%), 2 (10–50%), 3 (51–80%) and 4 (>80%). Multiplication of categories A and B resulted in an IRS ranging from 0 to 12 for each individual case. The raw expression scores were used for correlation analysis. For correlation with clinicopathological variables, cases that showed any expression of mTOR, 4EBP1, p-4EBP1, p-S6K and p-eIF4E (IRS 1–12) were scored as positive; cases without expression (IRS 0) were scored as negative.

**Statistical analysis**

Statistical analyses were carried out with SPSS 16.0 and GraphPad Prism 4.0. The significance of correlations between mTOR, 4EBP1, p-4EBP1, p-S6K and p-eIF4E staining patterns and clinicopathological data was tested by Fisher’s exact test and χ² test for trends. The significance of correlations of mTOR, 4EBP1, p-4EBP1, p-S6K and p-eIF4E expression scores in primary tumours and their corresponding lymph node and distant metastases was assessed by the Wilcoxon test for paired sample analysis. The correlation of mTOR, 4EBP1, p-4EBP1, p-S6K and p-eIF4E expression scores with each other and with proliferation indices was done by Spearman’s
rank order correlation. Distribution of mTOR, 4EBP1, p-4EBP1, p-S6K and p-eIF4E expression scores in dependence of tumour location was assessed by the Mann–Whitney U test. Differences in the percentages of Ki-67-positive cells in primary and metastatic tumours were investigated by the unpaired t-test and the Mann–Whitney U test.

The probability of differences in overall survival as a function of time was determined using the Kaplan–Meier method, with a log-rank test to probe for significance. P values <0.05 were considered significant.

Results

Expression patterns of mTOR, 4EBP1, p-4EBP1, p-S6K and p-eIF4E in GEP-NET

Cytoplasmic mTOR expression was found in 60 (61.2%) out of 98 tumours available for analysis. No nuclear immunostaining was observed. The intensity of immunostaining ranged from weak to strong and was fairly homogenous throughout a given tumour (Fig. 1). mTOR expression was significantly higher in foregut tumours than in midgut tumours (P=0.005, Fig. 2); this was also true when stage was included in the analysis (data not shown). There was no significant difference between mTOR expression in gastric, duodenal and pancreatic tumours (P=0.096, data not shown). However, while gastric and pancreatic tumours showed the same prevalence of mTOR positivity (~67%), duodenal tumours were less likely to be positive (16.7%).

Cytoplasmic 4EBP1 immunopositivity was noted in 91 (92.9%) out of 98 tumours investigated (Fig. 1). A very faint nuclear staining was detected in some cases, which might correspond to the nuclear localisation of the phosphorylated protein (see below). However, nuclear staining was too weak to allow for a quantitative evaluation of this staining pattern. Expression of 4EBP1 was significantly higher in foregut tumours than in their midgut counterparts (P<0.001, Fig. 2), which again was independent from tumour stage (data not shown). No significant differences in expression were found when gastric, duodenal and pancreatic tumours were compared (P=0.591, data not shown).

Phosphorylated 4EBP1 was located either in the cytoplasm or in the nucleus in 79 (79.8%) and 68 (68.7%) cases respectively (Fig. 1). Both cytoplasmic and nuclear positivity were significantly more likely to be found in foregut than in midgut tumours (P<0.001 for both correlations). This finding was also valid after differences in stage were taken into account (data not shown). Gastric, duodenal and pancreatic tumours showed no significant differences in the expression of cytoplasmic (P=0.443) and nuclear p-4EBP1 (P=0.105). However, pancreatic tumours showed a lower percentage of positive cases for nuclear expression (67.7%) when compared with duodenal (83.3%) and gastric (100%) tumours (data not shown).

| Table 3 Correlation of mTOR, 4EBP1, p-4EBP1, p-S6K and p-eIF4E expression in gastroenteropancreatic neuroendocrine tumours |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
|                | mTOR score | 4EBP1 score | Cytoplasmic score | Nuclear score | p-S6K score |
| 4EBP1 score    | r=0.322     | P=0.001      |                 |                |                |
| Cytoplasmic p-4EBP1 score | r=0.402 | P<0.001 | r=0.632 | P<0.001 |                |
| Nuclear p-4EBP1 score | r=0.260 | P=0.009 | r=0.406 | P<0.001 | r=0.642 |
| p-S6K score    | r=0.187     | P=0.067      | r=0.443         | P<0.001      | r=0.281 |
|               | r=0.259     | P=0.010      | r=0.162         | P=0.013      | r=0.374 |

A Kasajima et al.: mTOR in neuroendocrine tumours
Phosphorylated S6K (p-S6K) was exclusively found in the cytoplasm of tumour cells (Fig. 1). In total, 56.7% of tumours were positive for activated S6K to varying degrees (Tables 1 and 2). Similar to mTOR and 4EBP4, p-S6K expression was higher in foregut than in midgut tumours ($P<0.001$, Fig. 2) in a stage-independent manner. There was no significant difference in the expression of p-S6K between gastric, duodenal and pancreatic tumours ($P=0.786$, data not shown).

p-eIF4E was observed in 79.4% of tumours and varied considerably from case to case (Fig. 1, Tables 1 and 2). Again, expression was significantly higher in tumours from foregut when compared with tumours from midgut origin ($P<0.002$, Fig. 2). With respect to specific foregut locations, the number of positive cases did not show a relevant variation ($P=0.983$, data not shown).

Overall mTOR expression significantly correlated with 4EBP1, cytoplasmic and nuclear p-4EBP1 expression as well as with p-eIF4E expression ($P<0.01$ for all comparisons). The correlation coefficients ($r$) indicated a modest to fairly strong degree of interaction (Table 3). mTOR was associated with p-S6K as well; however, the association was weak ($r=0.187$) and failed to show statistical significance ($P=0.067$, Table 3).

As normal reference control, mTOR pathway component expression was investigated in a set of normal pancreatic tissues including adjacent stromal and inflammatory cells. These stainings revealed stable expression of several of the proteins in a distinct set of normal cells (e.g. lymphocytes). The respective results are summarised in Supplementary Table 1, see section on supplementary data given at the end of this article.

Correlation of mTOR, 4EBP1, p-4EBP1, p-S6K and p-eIF4E expression with proliferation indices

Foregut tumours showed a higher proliferative activity than midgut tumours (mean foregut: 11% Ki-67-positive cells, mean midgut: 5% Ki-67-positive cells, $P=0.002$). This was also found when only stage IV tumours were compared ($P<0.001$).

By trend, Ki-67 staining was higher in nodal (mean primary: 3.1%, mean nodal metastasis: 4.2%) and distant metastases (mean primary: 3.3%, mean distant metastasis: 8.3%) when compared with the corresponding primary tumours. These differences were statistically significant in parametric tests for both comparisons ($P<0.001$) but only for the comparison of primary tumour and distant metastasis in non-parametric tests ($P=0.024$).

Correlation of mTOR, 4EBP1, p-4EBP1, p-S6K and p-eIF4E expression with clinicopathological variables

In foregut, mTOR expression was significantly higher in tumours with distant metastasis ($P=0.035$; Table 1). No other correlations of the expression of mTOR, 4EBP1, p-4EBP1, p-S6K and p-eIF4E with clinicopathological variables in either foregut or midgut tumours were evident (Tables 1 and 2).

Correlation of mTOR, 4EBP1, p-4EBP1, p-S6K and p-eIF4E expression in the primary tumour and in corresponding lymph node and distant metastases

We investigated the expression of mTOR, 4EBP1, p-4EBP1, p-S6K and p-eIF4E in matched pairs of
primary tumours, nodal and distant metastases of GEP-NET (Fig. 4). There was a tendency towards lower mTOR expression in distant metastasis when compared with the respective primary tumours; however, this correlation was only of borderline significance ($P=0.07$). In addition, metastatic nodal (only cytoplasmic p-4EBP1) and distant tumour seeds usually showed slightly higher expression of phosphorylated 4EBP1, S6K and eIF4E when compared with the corresponding primary tumour, indicating higher activity of the mTOR pathway in metastatic tumours. However, this association was only found to be significant for cytoplasmic p-4EBP1 and nodal spread ($P=0.02$) and was of borderline significance for nuclear p-4EBP1 and distant spread ($P=0.07$).

Correlation of mTOR, 4EBP1, p-4EBP1, p-S6K and p-eIF4E expression with survival

A probatory survival analysis in the homogenous subgroup of patients with stage IV midgut tumours ($n=39$) revealed that neither mTOR ($P=0.329$) nor 4EBP1 ($P=0.186$) or p-eIF4E ($P=0.521$) expression had an impact on NET-related death in univariate survival analysis in this group of patients (Fig. 5).

Those patients whose tumours showed cytoplasmic p-4EBP1 expression had a trend towards longer disease-specific survival than those patients without activation of 4EBP1 ($P=0.055$). Interestingly, patients with activated S6K in their tumours had a significantly shortened disease-specific survival ($P=0.048$, Fig. 5). Neither grade ($P=0.764$) as a correlate for tumour aggressiveness nor treatment ($P=0.148$) had an impact on survival in this stage IV midgut patient cohort.

Discussion

In this study, we report a differential expression of mTOR, 4EBP1, phosphorylated 4EBP1, phosphorylated S6K and phosphorylated eIF4E in a large cohort of GEP-NET. Expression levels of mTOR as well as activation of its downstream targets were higher in foregut tumours than in midgut tumours, indicating a higher activity of the mTOR pathway in the former. This increase in activity was accompanied by a higher proliferative capacity of foregut tumours when compared with midgut tumours. Foregut tumours with distant metastases showed strong mTOR expression, and metastatic tumours in general showed slightly higher mTOR pathway activation indicated by enhanced phosphorylation of 4EBP1 as well as by enhanced phosphorylation of S6K and eIF4E. Interestingly, those stage IV midgut patients with activated S6K had a reduced disease-specific survival, while this was not true for other downstream effectors or mTOR itself.

The detection of p-4EBP1 in the nucleus by us and other groups both in vitro and in vivo is interesting (Zhou et al. 2004, Castellvi et al. 2006, Rojo et al. 2007, Rong et al. 2008). It has been demonstrated that the target of 4EBP1, eIF4E, has functions as a nuclear regulator of the export of several RNAs involved in proliferation and cell growth (Culjkovic et al. 2007). The presence of 4EBP1 in the nucleus has been proposed to provide a means to regulate the release of eIF4E from the nucleus and may thus prevent the untimely export of eIF4E bound mRNAs (Missiaglia et al. 2010). The relevance of this mechanism with respect to carcinogenesis has to be elucidated.

Recently, researchers have begun to focus on the mTOR pathway in GEP-NET, since treatment of metastasized NETs with the mTOR inhibitor everolimus in combination with octreotide showed promising results in phase II clinical studies (Yao et al. 2008b, 2010). In addition, the mTOR pathway plays a central role in the tumourigenesis of familial cases as well as in the sporadic cases of NETs. The notion that this pathway is of importance in this tumour entity has
further been substantiated by results of a high-throughput RNA expression analysis of pancreatic NETs in which the upstream inhibitors of mTOR, TSC2 and PTEN were found to be downregulated (Missiaglia et al. 2010). In addition, mTOR inhibition by rapamycin has been shown to significantly reduce NETs cell growth in vitro and in vivo (Moreno et al. 2008). This might be due to an induction of growth arrest in G0/G1 phase and enhanced apoptosis (Zitzmann et al. 2007). Furthermore, it has been proposed that deactivation of the AKT–mTOR kinase axis is responsible for this effect (Grozinsky-Glasberg et al. 2008). These in vitro results are in line with our findings that mTOR expression as well as downstream activation of 4EBP1, eIF4E and S6K correlates with proliferation in GEP-NET.

Most recently, in analogy to our work in GEP-NET, a large study on the expression of mTOR pathway components in lung NETs has been published in this journal (Righi et al. 2010). The authors reported an overexpression of p-4EBP1 in high-grade tumours, in contrast to p-mTOR and p-S6K, which were strongly expressed in low-grade tumours. In addition, in one recently published study on gastrointestinal NETs, phosphorylated mTOR, p-4EBP1 and p-S6K expression as well as several other factors were used to subclassify NET into novel potentially biological important subgroups (Iida et al. 2010). However, a correlation of the respective proteins with clinico-pathological variables and outcome has not been reported. Besides this, just one study on the expression of p-mTOR, which included only 20 GEP-NET (Shida et al. 2010) and in which the authors reported enhanced p-mTOR expression in poorly differentiated tumours, has been published. In our study, we did not find a straightforward correlation of either grouped mTOR expression or mTOR activity (as indicated by phosphorylation of 4EBP1) with tumour grade. However, we found an association of the expression of these proteins with the proliferation index, which in the novel grading scheme for GEP-NET is the central classifier for tumour grade.

mTOR expression and activity have been evaluated in a broad variety of human tumours, including most of the major tumour types, namely endometrial (Darb-Esfahani et al. 2009), esophageal (Boone et al. 2008), renal (Campbell et al. 2008), colorectal (Tampellini et al. 2007), prostate (Kremer et al. 2006), liver (Sahin et al. 2004), breast (Zhou et al. 2004, Rojo et al. 2007), lung (Anagnostou et al. 2009) and ovarian (Noske et al. 2008) cancer as well as glioblastoma (Pelloski et al. 2006). In all tumour entities, mTOR was either upregulated and/or activated in the tumour tissue when compared with the

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**Figure 5** Correlation of mTOR, 4EBP1, p-4EBP1, p-S6K and p-eIF4E expression with survival in stage IV midgut NET. No significant differences in disease-specific survival were observed in dependence of mTOR (A) and 4EBP1 (B), cytoplasmic (C) and nuclear (D) p-4EBP1 as well as p-eIF4E (F) positivity. In contrast, patients whose tumours expressed phosphorylated S6K (E) had a reduced disease-specific survival time. *P* values were calculated with a log-rank test.
corresponding tissue of origin. In addition, in some tumour entities, mTOR activity was linked to compromised patient prognosis. However, an association of the activated mTOR pathway with a better patient prognosis has been reported (Noske et al. 2008, Anagnostou et al. 2009) as well. In one study on bronchial NETs, no prognostic impact of mTOR pathway components was reported (Righi et al. 2010). We found that although mTOR expression itself was not associated with differences in patient prognosis, the detection of activated S6K confers a poor prognosis in stage IV midgut NETs. However, since this very homogenous subgroup of patients comprised only 39 cases, our results with respect to a possible impact of p-S6K positivity on survival must clearly be confirmed in much larger study cohorts.

In summary, we found that expression and activity of mTOR were strongly dependent on primary tumour location and metastatic status in GEP-NET. Expression as well as activation of mTOR pathway components was associated with enhanced proliferative capacity. Since everolimus, a small molecule targeting mTOR, proved to be effective in this tumour type and since this very homogenous subgroup of patients comprised only 39 cases, our results with respect to a possible impact of p-S6K positivity on survival must clearly be confirmed in much larger study cohorts.

Supplementary data

This is linked to the online version of the paper at http://dx.doi.org/10.1677/ERC-10-0126.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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References


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