necessarily complete [6]. Basal-like type breast carcinoma is currently considered not a synonym, but rather one of the prognostic factors for TNBC [6–9].

TNBC account for approximately 10–20% of the whole breast cancer cases and are associated with relatively early clinical relapses within 3 years, with frequent progression to distant metastasis, particularly, visceral metastasis and poor prognosis, although it is also true that they are sensitive to chemotherapy [10–13]. TNBC is generally considered the most difficult subtype to treat among these newly proposed subtypes of breast cancer because of the aggressive clinical behavior and the lack of current availability of specific targeted therapy such as selective ER modulators, aromatase inhibitors, trastuzumab, and lapatinib.

In addition, TNBC cases are also considered to be subclassified into different prognostic groups, because the patients associated with worse prognosis should be selected to receive the novel developing therapy. TNBC has been divided into subgroups based on the single prognostic biomarker, but further subclassification according to combination of valid biomarkers have not been reported in the literature at least in Asian patients of TNBC to the best of our knowledge. Therefore, in this study, we focused on tumor angiogenesis and proliferation that are major determinations of potential biological or clinical behavior of human breast cancer patients. We assessed Ki-67 labeling index (LI) and microvessel density (MVD) using CD31 staining, in addition to reported basal markers (CK5/6, CK14, and EGFR) and conventional clinicopathologic factors in TNBC. We also selected these markers from the standpoint that assessment tool should be used widely in clinical settings. We subsequently attempted to devise a scoring system which could reflect clinical outcome of the Japanese patients with TNBC patients in routine clinical practice.

Materials and methods

Patients

We identified 841 patients with primary operable breast cancer who underwent surgery at the Department of Surgery, Tohoku University Hospital (Sendai, Japan) between January 1998 and December 2007 and collected information through the breast cancer management database of the hospital. Among these 841 Japanese patients, 642 (76.3%) had hormone receptor positive, 138 (16.4%) had HER2-positive, 118 (14.0%) had triple receptor-negative tumors, and 6 (0.7%) lacked the receptor information. Of 118 TNBC above, patients with ductal carcinoma in situ apocrine carcinoma, medullary carcinoma were excluded from the present analysis, because these tumors have different biological and clinical features from those of invasive ductal TNBC [14–16], and we also excluded the cases that were not treated with adjuvant chemotherapy after the surgery in order to standardize backgrounds of the patients.

Characteristics of 102 triple negative patients examined in this study were summarized in Table 1. The median age of the patients was 56 years (range: 30–81 years). A number of 54 cases (53%) were classified as pT1, 36 (35%) pT2, and the others pT3/4. The median of pathological tumor size was 18 mm (range: 3–90 mm). Axillary node dissection was carried out in all the patients examined in this study. A total of 55 cases (54%) had a node-negative and 47 (46%) had a node-positive breast cancer. A number of 51 patients underwent breast conserving surgery who subsequently received postoperative radiotherapy against remaining breast tissue. Systemic adjuvant chemotherapy was administered to all the patients, and none of them was treated by hormonal therapy and HER2 targeting therapy.

Histopathology and immunohistochemistry

Hematoxylin–eosin (H&E)-stained glass slides were retrieved from surgical pathology files of Tohoku University Hospital,

Table 1 Characteristics of TNBC patients (n=102)

Characteristics	
Age, years	
Median (range)	56 (30–81)
≤50	39 (38%)
>50	63 (62%)
Pathological tumor size (mm)	
0–20	54 (53%)
21–30	18 (18%)
31–50	21 (20%)
51–	9 (9%)
Pathological node status	
0	55 (54%)
1–3	29 (28%)
4–9	8 (8%)
10–	10 (10%)
Distant metastasis	
Negative	102 (100%)
Positive	0 (0%)
Operation	
Mastectomy	51 (50%)
BCS	51 (50%)
Adjuvant systemic therapy	
Chemotherapy	102 (100%)
Endcrine therapy	0 (0%)
Anti-HER2 therapy	0 (0%)

BCS breast conserving surgery

Sendai, Japan, and reviewed in all of these 102 triple negative cases. The specimens were cut into 4-μm-thick sections and placed on glue-coated glass slides for immunohistochemistry (IHC). Tumor staging was based *on TNM Classification of Malignant Tumors*, 6th edn. by the International Union Against Cancer (UICC) [17]. Histological grades were assessed according to the criteria of Elston and Ellis [18]. We also identified the presence or absence of peritumoral vascular invasion (PVI), according to the report of Rosen and Obermann [19]. ER and PR status were immunostained by using monoclonal antibodies (codes 107925 and 102333, Roche Diagnostics, Basel, Switzerland), and nuclear staining of more than 1% was considered positive. HER2 status was evaluated by IHC (HercepTest, code K5204, Dako, Glostrup, Denmark) or by fluorescence in situ hybridization (FISH) that was used to calculate the gene copy ratio of HER2-to-CEP17 (the PathVysion HER2 DNA Probe Kit; Abbott, Chicago, IL). HER2 positivity was defined as 3+ receptor overexpression by IHC and/or as 2.2 or greater of a HER2-to-CEP17 ratio by FISH according to the American Society of Clinical Oncology/College of American Pathologists Guideline [20]. We performed IHC for Ki-67, CD31, EGFR, CK5/6, and CK14. Ki-67 LI was determined with MIB-1 monoclonal antibody (code M7240, Dako) through counting 1,000 tumor cells at the hot spots [21–24]. Microvessels were identified as the lumen lined by endothelial cells, complemented by IHC of endothelial cells with CD31 (code M0823, Dako). We counted microvessels at one high-power field (×200) as MVD after the areas with the greatest number of microvessels were selected at low magnification (×40 and×100) [25, 26]. EGFR was positive if the membrane of 10% or more carcinoma cells were stained by using representative monoclonal antibody (code K1492, Dako). CK5/6 and CK14 were interpreted positive if 10% or more carcinoma cells were positive in the cytoplasm with monoclonal antibodies (code M7237, Dako for CK5/6 and code NCL-LL002, Leica, Newcastle, UK for CK14) [5, 7, 8]. All of pathological diagnosis and staining assessments of individual cases were performed by two pathologists (Fig. 1).

Statistical methods

Breast cancer-specific survival (BCSS) was evaluated from the date of definitive surgery to that of the last follow-up or death due to breast cancer. Relapse-free survival (RFS) was defined as the length of time from the date of definitive surgery to the last follow-up or first event. Both BCSS and RFS were estimated by using the Kaplan–Meier methods, and the differences in survival curve between these two groups of the patients were assessed by the log–rank test. Cox proportional hazard model was used for univariate and multivariate analyses to evaluate each factor including pathological tumor size, pathological node status, PVI, histological grade, basal-like type, Ki-67 LI, and MVD. The hazard ratios (HRs) calculated for each significant factor in the multivariable model were defined as scores representing the risks of relapse and breast cancer specific death, and totaled or summated value of scores was subsequently defined as the risk score of TNBC patients. All analyses were performed using StatMate III® for Windows ver. 3.18 (ATMS, Tokyo, Japan). The results were considered significant with p value of <0.05. All statistical tests were two-sided.

Results

Table 2 shows the results of histopathological factors and biomarkers of TNBC patients. A number of 65 tumors (64%) had high-grade characteristics corresponding to histological grade III, and 39 tumors (38%) were associated with PVI. EGFR, CK5/6, and CK14 immunoreactivity was detected in 36 (35%), 60 (59%), and 42 cases (41%), respectively. TNBC was subsequently subdivided into the following groups: 67 (66%) basal-like cases which expressed one or more of the specific basal markers, EGFR, CK5/6, and CK14, and 35 (34%) nonbasal-like cases which did not express any of these markers above. The median value of Ki-67 LI was 37%, and 48 cases (47%) were assessed that cancer cells of more than 40% were stained. A number of 48 (47%) cases were positive for MVD at the cutoff point set at 20 as the optimal cutoff value near the median 19. During the median follow-up time of 68.5 months (range: 7–146 months), 37 patients experienced disease relapse and 28 patients died due to breast cancer. In univariate analysis, pathological tumor size with two cutoff points, i.e., 20 and 30 mm, pathological node status, PVI, the basal-like features, Ki-67 LI with two cutoff points, 30% and 40%, and the amount of microvessels were significantly correlated with poor prognosis or adverse clinical outcome of the patients.

Multivariate analysis and the subsequent risk score for TNBC

Age (≤50 vs. >50 years), pathological tumor size (≤20 vs. >20 mm or≤30 vs. >30 mm), pathological node status (negative vs. positive), PVI (negative vs. positive), histological grade (I, II vs. III), basal-like type (basal-like vs. nonbasal-like), Ki-67 LI (≤30% vs. >30% or≤40% vs. >40%), and MVD (≤20 vs. >20) were all assessed in multivariate analysis for RFS and BCSS. Among the factors evaluated above, the pathological nodal status was strongly associated with RFS (HR=2.99, 95% CI=1.45–6.19; p=0.003) and BCSS (HR=2.71, 95% CI=1.18–6.21;

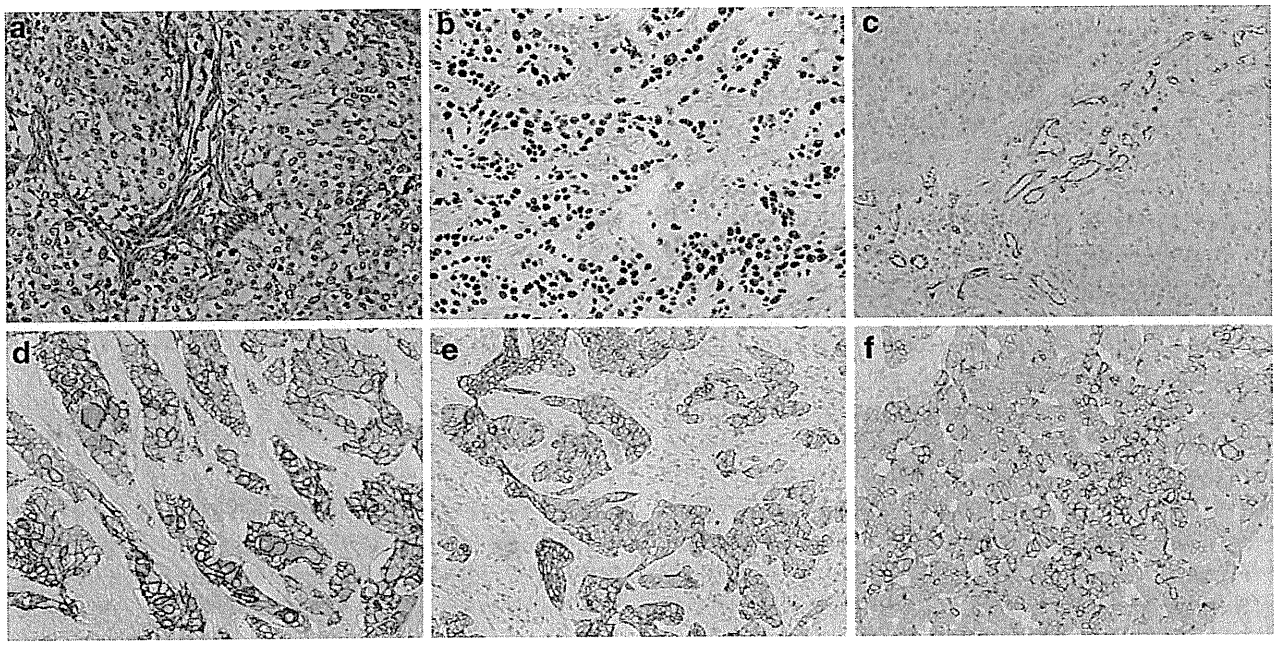

Fig. 1 Presentitative illustrations of H&E and immunohistochemical staining. **a** H&E, **b** high LI of Ki-67 staining, **c** high microvessel density by CD31 staining, **d** overexpression of epidermal growth factor receptor 1 (EGFR), **e** positive immunoreactivity of cytokeratin 5/6, and **f** positive staining of cytokeratin 14 (original magnification, ×200)

p=0.019). Pathological tumor size (≤30 vs. >30 mm) was also significantly associated with RFS (HR=2.23, 95% CI= 1.06–4.70; p=0.035) and BCSS (HR=2.63, 95% CI=1.13–6.12; p=0.025). Basal-like type was also significantly associated with RFS (HR=2.76, 95% CI=1.14–6.66; p= 0.024) and BCSS (HR=3.02, 95% CI=1.05–8.67; p= 0.040). Ki-67 LI (tentatively classified into two groups: ≤40% vs. >40%) was also significantly associated with RFS (HR=2.44, 95% CI=1.20–4.96; p=0.014) and BCSS (HR=2.68, 95% CI=1.15–6.26; p=0.023). MVD was also significantly associated with RFS (HR=2.32, 95% CI= 1.09–4.94; p=0.029) and BCSS (HR=2.44, 95% CI=1.02–5.86; p=0.046). Age, pathological tumor size (≤20 vs. >20 mm), PVI, histological grade, Ki-67 LI (≤30% vs. >30%) were not independently associated with RFS and BCSS (Table 3).

After these statistical analyses, we identified the following five significant factors which were significantly correlated with prognosis or clinical outcome of TNBC patients in multivariate analysis (pathological tumor size, pathological node status, basal-like type, Ki-67 LI, and MVD). We then further subclassified TNBC according to the five statistically significant risk factors described above. We applied scores reflecting HR points to each risk factor using a method that was presented for the prognostic models in cardiovascular disease as well as preoperative endocrine prognostic index (PEPI) for breast cancer cases built by this approach with some modifications [27, 28]. The procedure of scoring was summarized in Table 4. TNBC patients were classified into three groups by the risk score representing totaled value or summed score of each risk factor. Patients with score 0–3 are in low-risk group (A), score 4–7 in intermediate-risk group (B), and score 8–10 in high-risk group (C). These three groups had statistically different risks of relapse and breast cancer specific death, respectively (RFS: A vs. B, p<0.001; A vs. C, p < 0.001; B vs. C, p=0.013; BCSS: A vs. B, p = 0.025; A vs. C, p<0.001; B vs. C, p=0.005; Figs. 2 and 3).

Discussion

TNBC is generally considered to be associated with aggressive clinical behavior partly due to the limitations of the specific therapies currently available in clinical practice. However, it is also true that a marked heterogeneity exists in terms of clinical outcome or prognosis and response to various chemotherapeutic agents among TNBC patients [6, 10–13]. For instance, in putative low-risk patients of TNBC, the current standard chemotherapy regimens including anthracycline and taxanes have been usually considered somewhat effective [29–31]. However, it is also true that the new therapeutic strategy is being required to improve the prognosis of high-risk patients with TNBC. BRCA1 dysfunction and deficiency of DNA double-strand break repair were also reported to be detected in triple negative tumors, and these tumors may be more sensitive to poly(ADP-ribose) polymerase (PARP) inhib-

Table 2 Histopathological factors and biomarkers of TNBC patients (n=102)

Histopathological factors and biomarkers	
Histological grade	
I	5 (5%)
II	32 (31%)
III	65 (64%)
PVI	
Negative	63 (62%)
Positive	39 (38%)
EGFR	
Negative	66 (65%)
Positive	36 (35%)
CK5/6	
Negative	42 (41%)
Positive	60 (59%)
CK14	
Negative	60 (59%)
Positive	42 (41%)
Ki-67 labeling index	
0–15%	18 (18%)
16–30%	25 (24%)
31–40%	11 (11%)
41–100%	48 (47%)
Microvessel density	
≤20	54 (53%)
>20	48 (47%)

PVI:peritumoral vascular invasion, EGFR:epidermal growth factor receptor 1

itors [32, 33]. Ongoing phase III trial for PARP inhibitor is particularly expected to contribute to the treatment of TNBC. In addition, vascular endothelial growth factor (VEGF), recognized as the major angiogenic factor, was also abundantly detected in TNBC, and its levels have been demonstrated to be correlated with the survival time in TNBC [34–37]. In addition, several ongoing clinical trials suggest that bevacizumab could demonstrate the activity in patients with ER- and PR-negative cancer, most of them were also HER2-negative at subset analysis [31, 38, 39]. Therefore, considering these recent development of target-specific therapy in the patients with breast cancer, TNBC is currently being required to be classified pluralistically by the several prognostic factors in order to provide the most appropriate therapy to the patients, and these factors should be available in routine clinical settings considering the increment of breast cancer incidence in many parts of the world including the countries of Asia.

Therefore, in this study, we first examined the possible value of Ki-67 LI as the tool to evaluate cell proliferation because this has been proven to be one of the most useful and widely used prognostic markers in breast cancer despite some problems in procedures, interpretation, and reproducibility [21–24]. However, the analysis between Ki-67 LI in carcinoma cells and clinical outcome or behavior has not been reported in details in TNBC patients. The results of our study demonstrated that RFS and BCSS were categorized at two cutoff points of 30% and 40% and the patients whose LI was more than 40% were significantly associated with aggressive clinical behavior. High Ki-67 expression in carcinoma cells has been reported a prognostic factor for

Table 3 Multivariate analysis of clinicopathological factors and biomarkers for Relapse-free survival and Breast cancer-specific survival

Clinicopathological factors and biomarkers	Relapse-free survival				Breast cancer-specific survival			
	HR	95%CI		*P* value	HR	95%CI		*P* value
		Lower	Upper			Lower	Upper	
Age (>50 vs≤50)	0.93	0.46	1.91	0.85	0.62	0.27	1.43	0.260
Pathological tumor size								
≤20 vs >20 (mm)	1.64	0.81	3.32	0.17	2.05	0.89	4.72	0.091
≤30 vs >30 (mm)	2.23	1.06	4.70	0.04	2.63	1.13	6.12	0.025
Pathological node status (negative vs positive)	2.99	1.45	6.19	<0.01	2.71	1.18	6.21	0.019
PVI (negative vs positive)	1.43	0.71	2.90	0.32	1.05	0.46	2.38	0.905
Histological grade (I,II vs III)	1.01	0.65	1.52	0.97	1.17	0.48	2.83	0.728
Basal type (non basal-like vs basal-like)	2.76	1.14	6.66	0.02	3.02	1.05	8.67	0.040
Ki-67 labeling index								
≤30% vs >30%	1.82	0.84	3.91	0.13	2.34	0.91	6.05	0.079
≤40% vs >40%	2.44	1.20	4.96	0.01	2.68	1.15	6.26	0.023
Microvessel density (≤20 vs >20)	2.32	1.09	4.94	0.03	2.44	1.02	5.86	0.046

PVI peritumoral vascular invasion, *HR* hazard ratio, *CI* confidence interval

Table 4 The risk score for TNBC patients

Clinicopathological factors and biomarkers	Relapse-free survival		Breast cancer-specific survival	
	HR	Score	HR	Score
Pathological tumor size				
≤30 mm	–	0	–	0
>30 mm	2.23	2	2.63	3
Pathological node status				
Negative	–	0	–	0
Positive	2.99	3	2.71	3
Basal type				
Non basal-like	–	0	–	0
Basal-like	2.76	3	3.02	3
Ki-67 labeling index				
≤ 40%	–	0	–	0
> 40%	2.44	2	2.68	3
Microvessel density				
≤ 20	–	0	–	0
> 20	2.32	2	2.44	2

Hazard Ratio (HR) in the range of 1–2 receives 1 risk score, HR in the 2–2.5 range receives 2 risk scores, HR greater than 2.5 receives 3 risk scores. The Risk Score for each patient is the sum of all the risk scores accumulated from the five prognostic factors

breast cancer in many studies, but standard threshold in daily practice has not been established yet, partly due to various inert problems described above. In meta-analysis of Ki-67 LI in breast carcinoma patients reported in the literature, some studies used 10% as the cutoff (arbitrary value), whereas others have selected mean, median, and the optimal cutoff values [24]. The choice of cutoff points was also reported to depend on the various clinical objectives [24]. In our present studies of Ki-67 LI of TNBC, the cutoff point corresponding to high proliferation group was set at 40% as the optimal cutoff value near the median (Ki-67 LI: median; 37%) [22, 24], and Ki-67 LI determined in this manner turned out to be significant prognostic factor of the patients with TNBC.

We then evaluated the status of neovascularization in these TNBC cases using MVD. The analysis of MVD in resected tissue specimens has been generally reported to be a useful marker of tumor angiogenesis [40–45]. Meta-analysis of angiogenesis has been reported previously in 87 studies in the patients with breast cancer. Among these reported studies, 25 studies demonstrated the significant association between MVD and eventual survival of the patients. It is true, however, that the patients characteristics, antibodies, staining techniques, counting procedures, and

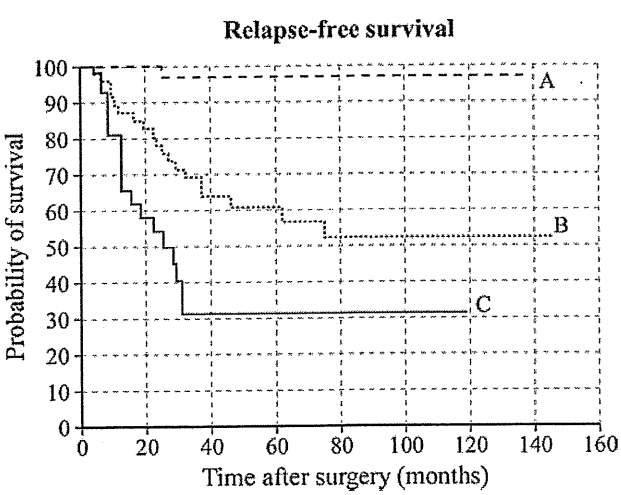

Fig. 2 Relapse-free survival in **a** low-risk group (score: 0–3, n=29), **b** intermediate-risk group (score: 4–7, n=48), and **c** high-risk group (score: 8–10, n=25) by the risk score system for TNBC (log–rank test: **a** vs. **b**, p<0.001; **A** vs. **C**, p < 0.001; **b** vs. **c**, p=0.013)

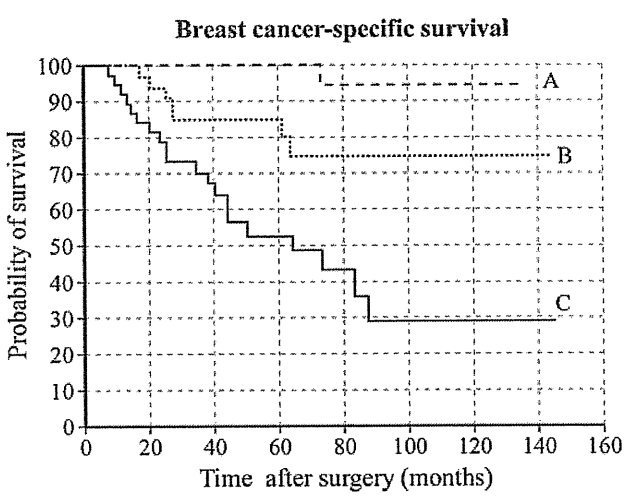

Fig. 3 Breast cancer-specific survival in **a** low-risk group (score: 0–3, n=29), **b** intermediate-risk group (score: 4–7, n=34), and **C** high-risk group (score: 8–10, n = 39) by the risk score system for TNBC (log–rank test: **a** vs. **b**, p=0.025; **a** vs. **c**, p<0.001; **b** vs. **c**, p=0.005)

selection of the cutoff levels of MVD assessment enormously varied among these reported studies, and it is very difficult to compare the results among individual studies [41]. In our present study, the statistically significant association was detected between high MVD and RFS, BCSS in TNBC patients using CD31, one of the gold standard markers of analyzing vascularity, with the optimal cutoff value near the median. However, standardization of assessment technique for MVD is required to obtain the vascularity in a more reproducible manner [46], and it requires further investigations to consolidate the clinical value of MVD analysis as a prognostic marker of the patients with TNBC.

EGFR, CK5/6, and CK14 were reported as more specific biomarkers to define the basal-like subtype and to reflect the cancer survival as a result of surrogating gene expression profiles analysis [3–9]. The results of our present study also demonstrated that the basal-like subtype was correlated with worse RFS and BCSS as well as reported in the previous studies [7–9]. Not all of the special histological types present triple negative features, but in our present study, typical medullary and apocrine carcinomas were excluded from the analysis because these two special subtypes were usually associated with the better clinical outcome than invasive ductal TNBC, despite the fact that they shared triple negative features [14–16].

The results of our present study also clearly suggest which subgroup should be indicated and received benefit by the optional strategy, novel agents including PARP inhibitors, bevacizumab, cetuximab, platinum drugs, and others [31, 47]. The risk score is generally considered a useful index when clinicians make a decision to treat patients of any condition including cancer. In our presently proposed risk score model for patients categorized in the low-risk group, the additional therapy may not provide additional clinical benefits because of the potentially intrinsic better clinical outcome or of the not aggressive clinical course of the patients. However, a more aggressive treatment should be served to the intermediate and high-risk group at an early phase as possible for adjuvant settings. As an annotation, the number of patients in the indeterminate and high-risk group was different at the assessment of RFS from that of BCSS, because HR points and risk scores were variable between RFS and BCSS in some of prognostic factors.

In addition, it is also important to note that this scoring system which we proposed in this report can be performed only by immunohistochemical techniques available for the great majority of diagnostic pathology laboratories. Therefore, an assessment of the risk score for TNBC can be reasonably postulated to be incorporated in any surgical pathology. The limitations of this study, however, include the retrospective nature of analysis, the strength of relatively short follow-up time for the patients with breast cancer (median: 68.5 months), and the availability of only one ethnic group, Japanese. Further studies with prospective validation, larger sample size, and longer follow-up are required to confirm this scoring system and to improve the model as a tool of classification of TNBC.

In conclusion, the results of our present study firstly demonstrated that TNBC could be further subclassified into three different groups according to the risk score system evaluating the following five prognostic variables (pathological tumor size, pathological node status, basal-like type, Ki67 LI, and MVD). Such a classification, which can be performed in diagnostic pathology laboratory, can be useful as a decision-making tool for triple negative patients.

Acknowledgments We wish to thank Yayoi Takahashi, MT, for her excellent technical assistance. This work was partly supported by a grant-in-aid for researches on biological feature and therapeutic strategy of TNBC (no. 22591421) from the Japanese Ministry of Education, Culture, Sports Science and Technology.

Conflicts of interest None

References

1. Perou CM, Sorlie T, Eisen MB et al (2000) Molecular portraits of human breast tumors. Nature 406:747–752
2. Sorlie T, Perou C, Tibshirani R et al (2001) Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. Proc Natl Acad Sci USA 98(19):10869–10874
3. Nielsen TO, Hsu FD, Jensen K et al (2004) Immunohistochemical and clinical characterization of the basal-like subtype of invasive breast carcinoma. Clin Cancer Res 10(16):5367–5374
4. Livasy CA, Karaca G, Nanda R et al (2006) Phenotypic evaluation of the basal-like subtype of invasive breast carcinoma. Mod Pathol 19(2):264–271
5. Lerma E, Peiro G, Ramo'n T et al (2007) Immunohistochemical heterogeneity of breast carcinomas negative for estrogen receptors, progesterone receptors and Her2//neu (basal-like breast carcinomas). Mod Pathol 20(11):1200–1207
6. Reis-Filho JS, Tutt ANJ (2008) Triple negative tumours: a critical review. Histopathology 52:108–118
7. Rakha EA, El-Sayed ME, Green AR et al (2007) Prognostic markers in triple-negative breast cancer. Cancer 109(1):25–32
8. Rakha EA, Reis-Filho JS, Ian OE (2008) Basal-like breast cancer: a critical review. J Clin Oncol 26:2568–2581
9. Maggie CU-C, David V, Chris B et al (2008) Basal-like breast cancer defined by five biomarkers has superior prognostic value than triple-negative phenotype. Clin Cancer Res 14(5):1368–1376
10. Bauer KR, Brown M, Cress RD et al (2007) Descriptive analysis of estrogen receptor (ER)-negative, progesterone receptor (PR)-negative, and HER2-negative invasive breast cancer, the socalled triple-negative phenotype: a population-based study from the California cancer registry. Cancer 109(9):1721–1728
11. Carey LA, Dees EC, Sawyer L et al (2007) The triple negative paradox: primary tumor chemosensitivity of breast cancer subtypes. Clin Cancer Res 13(8):2329–2334
12. Rhee J, Han SW, Oh DY et al (2008) The clinicopathologic characteristics and prognostic significance of triple-negativity in node-negative breast cancer. BMC Cancer 8(1):307

13. Cornelia L, Chafika M, Kenneth RH et al (2008) Response to neoadjuvant therapy and long-term survival in patients with triple-negative breast cancer. J Clin Oncol 26:1275–1281
14. Anne VS, Nadège G, Carlo L et al (2007) Identification of typical medullary breast carcinoma as a genomic sub-group of basal-like carcinomas, a heterogeneous new molecular entity. Breast Cancer Res 9(2):1–15
15. Jocelyne J, Laetitia P, Laetitia R et al (2005) Typical medullary breast carcinomas have a basal/myoepithelial phenotype. J Pathol 207(3):260–268
16. Francois B, Pascal F, Nathalie C et al (2006) Gene expression profiling shows medullary breast cancer is a subgroup of basal breast cancers. Cancer Res 66(9):4636–4644
17. Sobin LH, Wittekind C (eds) (2002) TNM classification of malignant tumours, 6th edn. Wiley-Liss, New York, pp 131–141
18. Elston CW, Ellis IO (1991) Pathological prognostic factors in breast cancer: I. The value of histological grade in breast cancer: experience from a larger study with long-term follow-up. Histopathology 19:403–410
19. Rosen PP, Oberman H (1993) Tumors of the mammary gland. Armed Forces Institute of Pathology, Washington, DC
20. Wolff AC, Hammond MH, Schwartz JN et al (2007) American society of clinical oncology/college of American pathologists guideline recommendations for human epidermal growth factor receptor 2 testing in breast cancer. J Clin Oncol 25:118–145
21. Spyratos F, Ferrero-PM TM et al (2002) Correlation between MIB-1 and other proliferation marker clinical implications of the MIB-1 cutoff value. Cancer 94(8):2151–2159
22. Attiqa N, Nadeem Q, Georges V et al (2002) Prognostic factors in node-negative breast cancer a review of studies with sample size more than 200 and follow-up more than 5 years. Ann Surg 235:10–26
23. Ander U, Ian ES, Mitch D (2005) Proliferation marker Ki-67 in early breast cancer. J Clin Oncol 23:7212–7220
24. Azambuja E, Cardoso F, Castro G et al (2007) Ki-67 as prognostic marker in early breast cancer: a meta-analysis of published studies involving 12 155 patients. Br J Cancer 96:1504–1513
25. Weidner N, Semple JP, Welch WR et al (1991) Tumor angiogenesis and metastasis correlation in invasive breast carcinoma. N Engl J Med 324:1–8
26. Weidner N, Folkman J, Pozza F et al (1992) Tumor angiogenesis: a new significant and independent prognostic indicator in early-stage breast carcinoma. J Natl Cancer Inst 84:1875–1887
27. Morrow DA, Antman EM, Charlesworth A et al (2000) TIMI risk score for ST-elevation myocardial infarction: a convenient, bedside, clinical score for risk assessment at presentation: an intravenous nPA for treatment of infarcting myocardium early II trial substudy. Circulation 102(17):2031–2037
28. Matthew JE, Yu T, Jingqin L et al (2008) Outcome prediction for estrogen receptor-positive breast cancer based on postneoadjuvant endocrine therapy tumor characteristics. J Natl Cancer Inst 100:1380–1388
29. Wang S, Yang H, Tong F et al (2009) Response to neoadjuvant therapy and disease free survival in patients with triple-negative breast cancer. Gan To Kagaku Ryoho 36(2):255–258
30. Hugh J, Hanson J, Cheang MC et al (2009) Breast cancer subtypes and response to docetaxel in node-positive breast cancer: use of an immunohistochemical definition in the BCIRG 001 trial. J Clin Oncol 27(8):1168–1176
31. Edith A, Perez A, Aubrey T et al (2010) Adjuvant therapy of triple negative breast cancer. Breast Cancer Res Treat 120:285–291
32. Bryant HE, Schultz N, Thomas HD et al (2005) Specific killing of BRCA2-deficient tumours with inhibitors of poly (ADP-ribose) polymerase. Nature 434(7035):913–917
33. Ratnam K, Low JA (2007) Current development of clinical inhibitors of poly(ADP-ribose) polymerase in oncology. Clin Cancer Res 13(5):1383–1388
34. Yamamoto Y, Toi M, Kondo S et al (1996) Concentrations of vascular endothelial growth factor in the sera of normal controls and cancer patients. Clin Cancer Res 2:821–826
35. Adams J, Carder PJ, Downey S et al (2000) Vascular endothelial growth factor (VEGF) in breast cancer: comparison of plasma, serum, and tissue VEGF and microvessel density and effects of tamoxifen. Cancer Res 60:2898–2905
36. Foekens JA, Peters HA, Grebenchtchikov N et al (2001) High tumor levels of vascular endothelial growth factor predict poor response to systemic therapy in advanced breast cancer. Cancer Res 61:5407–5414
37. Linderholm BK, Hellborg H, Johansson U et al (2009) Significantly higher levels of vascular endothelial growth factor (VEGF) and shorter survival times for patients with primary operable triple-negative breast cancer. Ann Oncol 20(10):1639–1646
38. Miller K, Wang M, Gralow J et al (2007) Paclitaxel plus bevacizumab versus paclitaxel alone for metastatic breast cancer. N Engl J Med 357:2666–2676
39. Ryan PD, Tung NM, Isakoff SJ et al (2009) Neoadjuvant cisplatin and bevacizumab in triple negative breast cancer (TNBC): safety and efficacy. J Clin Oncol 27 (Suppl). (Abstr 551)
40. Steinbjørn H, Dorthe AG, Flemming BS et al (2000) The prognostic value of angiogenesis by Chalkley counting in a confirmatory study design on 836 breast cancer patients. Clin Cancer Res 6:139–146
41. Bernard U, Patrick N, Michel C et al (2004) Microvessel density as a prognostic factor in women with breast cancer: a systematic review of the literature and meta-analysis. Cancer Res 64:2941–2955
42. Mohammed RAA, Green A, El-Shikh S et al (2007) Prognostic significance of vascular endothelial cell growth factors-A, -C and -D in breast cancer and their relationship with angio- and lymphangiogenesis. Br J Cancer 96:1092–1100
43. Rabab A, Ian O, Somaia E et al (2009) Lymphatic and angiogenic characteristics in breast cancer: morphometric analysis and prognostic implications. Breast Cancer Res Treat 113:261–273
44. Nair L, Barbara S, Daniella V et al (2009) Vessel density assessed by endoglin expression in breast carcinomas with different expression profiles. Histopathology 55:594–599
45. Nieto Y, Woods J, Nawaz F et al (2007) Prognostic analysis of tumour angiogenesis, determined by microvessel density and expression of vascular endothelial growth factor, in high-risk primary breast cancer patients treated with high-dose chemotherapy. Br J Cancer 97:391–397
46. Acenero MJ, Gonzalez JF, Gallego MG et al (1998) Vascular enumeration as a significant prognosticator for invasive breast carcinoma. J Clin Oncol 16:1684–1688
47. Ana B, Pilar E, Rosa Z et al (2010) Triple-negative breast cancer: molecular features, pathogenesis, treatment and current lines of research. Cancer Treat Rev 36:206–215

Endocrine-Related Cancer (2011) **18** 181–192

mTOR expression and activity patterns in gastroenteropancreatic neuroendocrine tumours

Atsuko Kasajima[1,3], Marianne Pavel[2], Silvia Darb-Esfahani[1], Aurelia Noske[4], Albrecht Stenzinger[1,6], Hironobu Sasano[3], Manfred Dietel[1], Carsten Denkert[1], Christoph Röcken[5], Bertram Wiedenmann[2] and Wilko Weichert[1,6]

[1]Institute of Pathology and [2]Department of Internal Medicine, Charité Universitätsmedizin, 10117 Berlin, Germany
[3]Department of Pathology, Tohoku University Graduate School of Medicine, 980-8575 Sendai, Japan
[4]Institute of Pathology, Universitätsspital Zürich, 8091 Zürich, Switzerland
[5]Institute of Pathology, Christian-Albrechts-Universität, 24105 Kiel, Germany
[6]Institute of Pathology, Ruprecht-Karls-Universität, Im Neuenheimer Feld 220/221, 69120 Heidelberg, Germany

(Correspondence should be addressed to W Weichert at Institute of Pathology, Ruprecht-Karls-Universität; Email: wilko.weichert@med.uni-heidelberg.de)

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.

Endocrine-Related Cancer (2011) **18** 181–192

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.* 2008*a*). 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 2008) 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.* 2008*b*, 2010) in a heterogeneous set of neuroendocrine neoplasms, and everolimus proved to be effective in the first place, especially in pancreatic

DOI: 10.1677/ERC-10-0126
1351–0088/11/018–181
Online version via http://www.endocrinology-journals.org

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 2004). mTORC1 itself is activated via the PI3K–AKT pathway partly through the deactivation of the tuberous sclerosis 1 (TSC1) and tuberous sclerosis 2 (TSC2) complexes (Gao *et al.* 2002). With respect to GEP-NET, this is interesting since patients with certain tumour syndromes with impaired TSC1/TSC2 function, such as tuberous sclerosis, are known to develop these neoplasms (Toumpanakis & Caplin 2008). The 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

Table 1 mTOR, 4EBP1, p-4EBP1, p-S6K and p-eIF4E expression in gastroenteropancreatic neuroendocrine tumours of the foregut and correlation with clinicopathological variables

Characteristics	All cases	mTOR negative	mTOR positive	*P* value	4EBP1 negative	4EBP1 positive	*P* value	p-4EBP1 cytoplasmic negative	p-4EBP1 cytoplasmic positive	*P* value	p-4EBP1 nuclear negative	p-4EBP1 nuclear positive	*P* value	p-S6K negative	p-S6K positive	*P* value	p-eIF4E negative	p-eIF4E positive	*P* value
All cases	47	14 (29.8%)	33 (70.2%)		1 (2.1%)	46 (97.9%)		2 (4.3%)	45 (95.7%)		11 (23.4%)	36 (76.6%)		10 (21.7%)	36 (78.3%)		7 (15.2%)	39 (84.8%)	
Stage				0.140[a]			0.884[a]			0.834[a]			0.359[a]			0.667[a]			0.216[a]
I	4 (8.9%)	2 (50%)	2 (50%)		0 (0%)	4 (100%)		0 (0%)	4 (100%)		1 (25%)	3 (75%)		2 (50%)	2 (50%)		2 (50%)	2 (50%)	
II	14 (31.1%)	4 (28.6%)	10 (71.4%)		0 (0%)	14 (100%)		0 (0%)	14 (100%)		2 (14.3%)	12 (85.7%)		0 (0%)	14 (100%)		0 (0%)	14 (100%)	
III	13 (28.9%)	7 (53.8%)	6 (46.2%)		1 (7.7%)	12 (92.3%)		2 (15.4%)	11 (84.6%)		3 (23.1%)	10 (76.9%)		3 (23.1%)	10 (76.9%)		1 (7.7%)	12 (92.3%)	
IV	14 (31.1%)	1 (7.1%)	13 (92.9%)		0 (0%)	14 (100%)		0 (0%)	14 (100%)		5 (35.7%)	9 (64.3%)		4 (28.6%)	10 (71.4%)		4 (28.6%)	10 (71.4%)	
Tumour stage				0.811[a]			0.717[a]			0.066[a]			0.848[a]			0.376[a]			0.700[a]
T1	7 (17.5%)	3 (42.9%)	4 (57.1%)		0 (0%)	7 (100%)		0 (0%)	7 (100%)		3 (42.9%)	4 (57.1%)		3 (42.8%)	4 (57.2%)		2 (33.3%)	4 (66.7%)	
T2	15 (37.5%)	4 (26.7%)	11 (73.3%)		0 (0%)	15 (100%)		0 (0%)	15 (100%)		2 (13.3%)	13 (86.7%)		2 (13.3%)	13 (87.7%)		(0%)	15 (100%)	
T3	5 (12.5%)	2 (40%)	3 (60%)		1 (20%)	4 (80%)		0 (0%)	5 (100%)		2 (40%)	3 (60%)		1 (20%)	4 (80%)		0 (0%)	5 (100%)	
T4	13 (32.5%)	4 (30.8%)	9 (69.2%)		0 (0%)	13 (100%)		2 (15.4%)	11 (84.6%)		4 (30.8%)	9 (69.2%)		2 (16.7%)	10 (83.3%)		3 (23.1%)	10 (76.9%)	
Nodal status				0.510[b]			0.386[b]			1.0[b]			0.724[b]			0.481[b]			1.0[b]
N0	28 (60.9%)	9 (32.1%)	19 (67.9%)		0 (0%)	28 (100%)		1 (3.6%)	27 (96.4%)		6 (21.4%)	22 (78.6%)		5 (18.5%)	22 (81.5%)		4 (14.3%)	24 (85.7%)	
N1	18 (39.1%)	5 (27.8%)	13 (72.2%)		1 (5.6%)	17 (94.4%)		1 (5.6%)	17 (94.4%)		5 (27.8%)	13 (72.2%)		5 (27.8%)	13 (72.2%)		3 (17.6%)	14 (82.4%)	
Metastasis				0.035[b]			1.0[b]			1.0[b]			0.225[b]			0.704[b]			0.190[b]
M0	32 (69.6%)	13 (40.6%)	19 (59.4%)		1 (3.1%)	31 (96.9%)		2 (6.3%)	30 (93.7%)		6 (20%)	24 (80%)		6 (19.3%)	25 (80.7%)		3 (9.7%)	28 (90.3%)	
M1	14 (30.4%)	1 (7.1%)	13 (92.9%)		0 (0%)	14 (100%)		0 (0%)	14 (100%)		5 (35.7%)	9 (64.3%)		4 (28.6%)	10 (71.4%)		4 (28.6%)	10 (71.4%)	
Grade				0.195[a]			/			0.622[a]			0.788[a]			0.263[a]			0.525[a]
G1	11 (35.5%)	3 (27.3%)	8 (72.7%)		0 (0%)	11 (100%)		0 (0%)	11 (100%)		2 (18.2%)	9 (81.8%)		3 (27.3%)	8 (72.7%)		2 (18.2%)	9 (81.8%)	
G2	16 (51.6%)	4 (25%)	12 (75%)		0 (0%)	16 (100%)		2 (12.5%)	14 (87.5%)		5 (31.2%)	11 (68.8%)		3 (18.8%)	13 (81.2%)		3 (18.8%)	13 (81.2%)	
G3	4 (12.9%)	3 (75%)	1 (25%)		0 (0%)	4 (100%)		0 (0%)	4 (100%)		0 (0%)	4 (100%)		0 (0%)	4 (100%)		0 (0%)	4 (100%)	

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.

[a]χ^2 test for trends.

[b]Fisher's exact test.

Table 2 mTOR, 4EBP1, p-4EBP1, p-S6K and p-eIF4E expression in enteric midgut tumours and correlation with clinicopathological variables

Characteristics	All cases	mTOR negative	mTOR positive	*P* value	4EBP1 negative	4EBP1 positive	*P* value	p-4EBP1 cytoplasmic negative	p-4EBP1 cytoplasmic positive	*P* value	p-4EBP1 nuclear negative	p-4EBP1 nuclear positive	*P* value	p-S6K negative	p-S6K positive	*P* value	p-eIF4E negative	p-eIF4E positive	*P* value
All cases	52	24 (47.1%)	27 (52.9%)		6 (11.8%)	45 (88.2%)		18 (34.6%)	34 (65.4%)		20 (38.5%)	32 (61.5%)		32 (62.7%)	19 (37.3%)		13 (25.5%)	38 (74.5%)	
Stage				0.609[a]			0.538[a]			0.234[a]			0.347[a]			0.681[a]			0.801[a]
I	0 (0%)	/	/		/	/		/	/		/	/		/	/		/	/	
II	2 (3.8%)	0 (0%)	1 (100%)		0 (0%)	2 (100%)		0 (0%)	2 (100%)		0 (0%)	2 (100%)		1 (50%)	1 (50%)		0 (0%)	2 (100%)	
III	11 (21.2%)	7 (63.6%)	4 (36.4%)		1 (9.1%)	10 (90.9%)		3 (27.3%)	8 (72.7%)		4 (36.4%)	7 (63.6%)		7 (63.6%)	4 (36.4%)		3 (27.3%)	8 (72.7%)	
IV	39 (75%)	17 (43.6%)	22 (56.4%)		5 (13.2%)	33 (86.8%)		15 (38.5%)	24 (61.5%)		16 (41%)	23 (59%)		24 (63.2%)	14 (36.8%)		10 (26.3%)	28 (73.7%)	
Tumour stage				0.727[a]			0.617[a]			0.879[a]			0.366[a]			0.145[a]			0.897[a]
T1	1 (2%)	1 (100%)	0 (0%)		0 (0%)	1 (100%)		1 (100%)	0 (0%)		1 (100%)	0 (0%)		1 (100%)	0 (0%)		0 (0%)	1 (100%)	
T2	8 (16%)	3 (42.9%)	4 (57.1%)		0 (0%)	8 (100%)		2 (25%)	6 (75%)		1 (12.5%)	7 (87.5%)		5 (62.5%)	3 (37.5%)		1 (12.5%)	7 (87.5%)	
T3	21 (42%)	9 (47.4%)	12 (52.6%)		4 (19%)	17 (81%)		7 (33.3%)	14 (66.7%)		7 (33.3%)	14 (66.7%)		16 (76.2%)	5 (23.8%)		7 (33.3%)	14 (66.7%)	
T4	20 (40%)	9 (45%)	11 (55%)		2 (10.5%)	17 (89.5%)		7 (35%)	13 (65%)		9 (45%)	11 (55%)		10 (52.6%)	9 (47.4%)		3 (15.8%)	16 (84.2%)	
Nodal status				0.547[b]			0.572[b]			0.233[b]			0.127[b]			0.697[b]			1.0[b]
N0	8 (15.7%)	3 (42.9%)	4 (57.1%)		0 (0%)	8 (100%)		1 (12.5%)	7 (87.5%)		1 (12.5%)	7 (87.5%)		4 (50%)	4 (50%)		3 (37.5%)	5 (62.5%)	
N1	43 (84.3%)	21 (48.8%)	22 (51.2%)		6 (14.3%)	36 (85.7%)		17 (39.5%)	26 (60.5%)		19 (44.2%)	24 (55.8%)		27 (64.3%)	15 (35.7%)		10 (23.8%)	32 (76.2%)	
Metastasis				0.749[b]			1.0[b]			0.329[b]			0.524[b]			1.0[b]			1.0[b]
M0	14 (26.9%)	7 (53.8%)	6 (46.2%)		1 (7.1%)	13 (92.9%)		3 (21.4%)	11 (78.6%)		4 (28.6%)	10 (71.4%)		8 (57.1%)	6 (42.9%)		3 (21.4%)	11 (78.6%)	
M1	38 (73.1%)	17 (44.7%)	21 (55.3%)		5 (13.5%)	32 (86.5%)		15 (39.5%)	23 (60.5%)		16 (42.1%)	22 (57.9%)		24 (64.9%)	13 (35.1%)		10 (27%)	27 (73%)	
Grade				0.773[a]			0.366[a]			0.353[a]			0.669[a]			0.307[a]			0.280[b]
G1	13 (36.1%)	8 (61.5%)	5 (38.5%)		3 (23.1%)	10 (76.9%)		4 (30.8%)	9 (69.2%)		5 (38.5%)	8 (61.5%)		10 (76.9%)	3 (23.1%)		4 (30.8%)	9 (69.2%)	
G2	21 (58.3%)	8 (38.1%)	13 (61.9%)		3 (14.3%)	18 (85.7%)		10 (47.6%)	11 (52.4%)		9 (42.9%)	12 (57.1%)		13 (61.9%)	8 (38.1%)		4 (19%)	17 (81%)	
G3	2 (5.6%)	2 (100%)	0 (0%)		0 (0%)	2 (100%)		1 (50%)	1 (50%)		0 (0%)	2 (100%)		1 (50%)	1 (50%)		0 (0%)	2 (100%)	

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] χ^2 test for trends.

[b] Fisher's exact test.

Instruments, Silver Spring, MD, USA). 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, 4EPB1, 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 χ^2 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

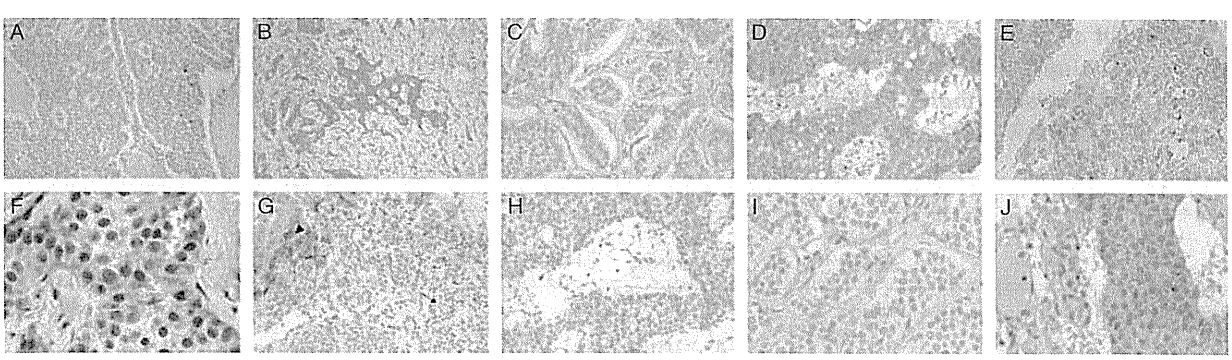

Figure 1 mTOR, 4EBP, p-4EBP1, p-S6K and p-eIF4E expression patterns in gastroenteropancreatic neuroendocrine tumours. (A/B) mTOR expression in GEP-NET. (A) An mTOR-negative tumour is shown. (B) Tumour with strong cytoplasmic mTOR positivity. (C/D) 4EBP1 in GEP-NET. Neuroendocrine tumours with weak (C) and strong (D) expression of 4EBP1. (E/F) p-4EBP1 expression in GEP-NET. (E) A tumour with strong cytoplasmic and without nuclear expression is depicted. In contrast, the tumour in (F) showed moderate cytoplasmic and strong nuclear positivity. (G/H) p-S6K in GEP-NET. While the tumour (arrow) in (G) was essentially negative for p-S6K, the tumour in (H) showed strong expression of the phosphorylated protein. Note strong expression in liver parenchyma (arrowhead in G). (I/J) p-eIF4E in GEP-NET. (I) A tumour without expression of p-eIF4E is depicted, while the tumour in (J) was scored as positive.

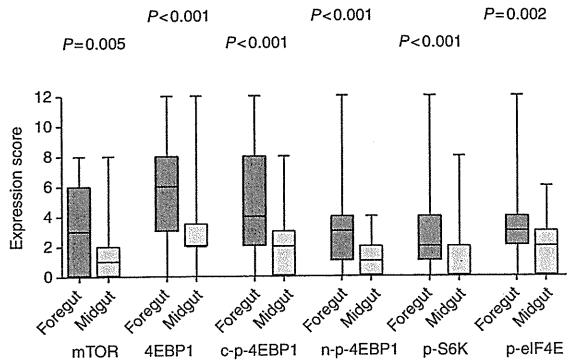

Figure 2 Expression of mTOR, 4EBP1, p-4EBP1, p-S6K and p-eIF4E in dependence of tumour location. Expression of mTOR, 4EBP1, p-4EBP1, p-S6K and p-eIF4E was higher in foregut than in midgut tumours. *P* values were calculated with the Mann–Whitney *U* test.

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 (Fig. 2, $P<0.001$ 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 p-4EBP1 score	Nuclear p-4EBP1 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	$r=0.260$ $P=0.009$	$r=0.406$ $P<0.001$	$r=0.642$ $P<0.001$		
p-S6K score	$r=0.187$ $P=0.067$	$r=0.443$ $P<0.001$	$r=0.281$ $P=0.005$	$r=0.239$ $P=0.019$	
p-eIF4E score	$r=0.346$ $P=0.001$	$r=0.646$ $P<0.001$	$r=0.259$ $P=0.010$	$r=0.162$ $P=0.113$	$r=0.374$ $P<0.001$

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 ($P=0.024$).

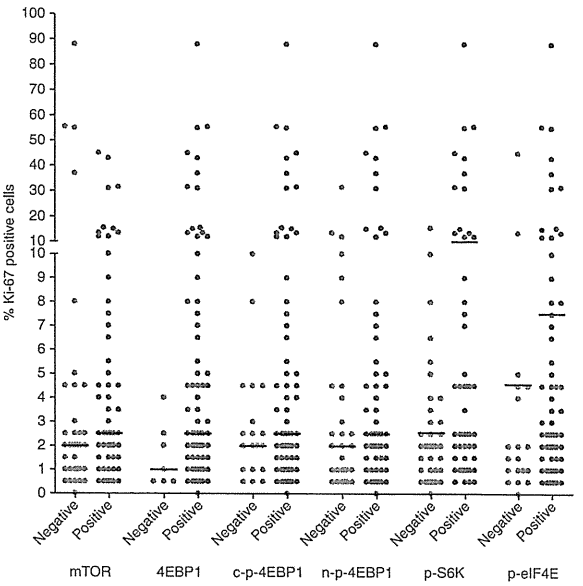

Figure 3 Proliferative activity in dependence of mTOR, 4EBP1, p-4EBP1, p-S6K and p-eIF4E expression in GEP-NET. Proliferative activity was higher in those tumours with stronger expression of mTOR, 4EBP1, p-4EBP1, p-S6K and p-eIF4E (details: see text).

Overall mTOR positivity was slightly but significantly higher in tumours with higher proliferative capacity ($r=0.213$, $P=0.038$). This correlation was also found for the expression of phosphorylated cytoplasmic and nuclear 4EBP1 ($r=0.238$, $P=0.020$ and $r=0.262$, $P=0.010$ respectively). Expression of 4EBP1 showed an even higher degree of correlation ($r=0.463$, $P<0.001$; Fig. 3). In addition, p-S6K ($r=0.364$, $P<0.001$) as well as p-eIF4E ($r=0.273$, $P=0.008$) expression was associated with higher proliferative capacity, as well (Fig. 3).

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

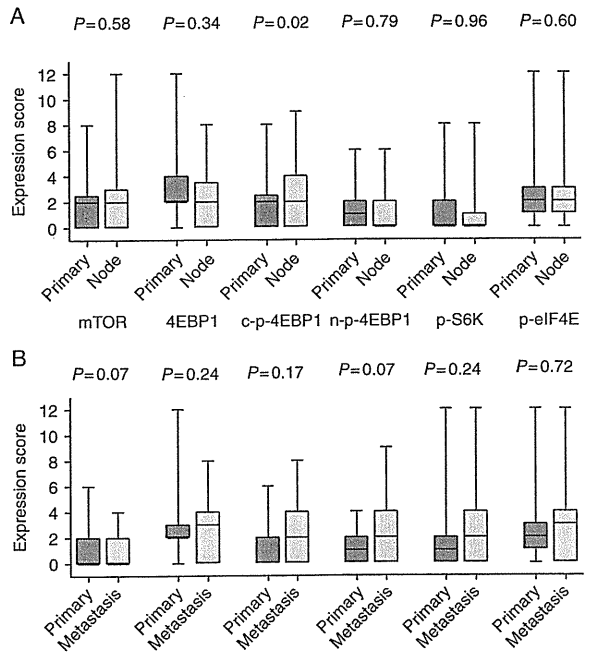

Figure 4 Correlation of mTOR, 4EBP1, p-4EBP1, p-S6K and p-eIF4E expression in primary tumours and corresponding lymph node and distant metastases. While mTOR expression is a bit lower in distant metastatic tumours when compared with the corresponding primaries, activation levels of 4EBP1 are usually higher in nodal and distant metastases and expression levels of p-S6K and p-eIF4E were higher in distant metastases. *P* values were calculated with the Wilcoxon test.

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.* 2008*b*, 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

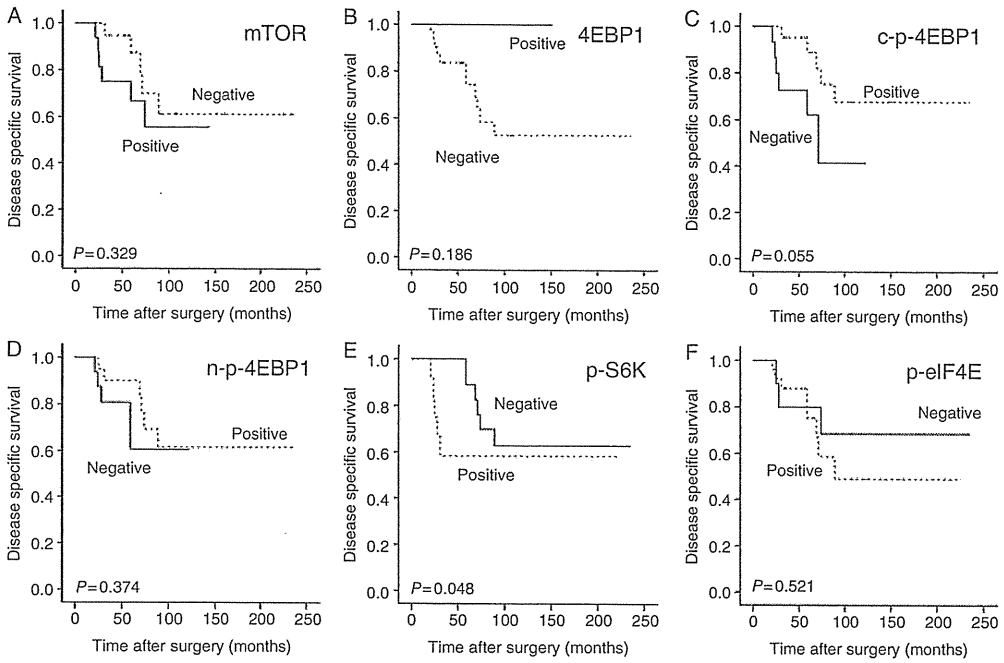

Figure 5 Correlation of mTOR, 4EBP1, p-4EBP1, p-S6K and p-eIF4E expression with survival in stage IV midgut NET. No significant differences in disease-specific survival were observed in dependence of mTOR (A) and 4EBP1 (B), cytoplasmic (C) and nuclear (D) p-4EBP1 as well as p-eIF4E (F) positivity. In contrast, patients whose tumours expressed phosphorylated S6K (E) had a reduced disease-specific survival time. *P* values were calculated with a log-rank test.

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 G_0/G_1 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

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 it has been shown that response to other mTOR inhibitors may vary in dependence of expression and/or activity of the target, we suggest an investigation of mTOR expression profiles and phosphorylation of downstream targets in future clinical trials with this inhibitor.

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.

Funding

This study was supported by a research stipend (Ernst von Leyden-Stipendium 2009) to A Kasajima by the Berliner Krebsgesellschaft.

Acknowledgements

We like to thank Lisa Glanz, Petra Wachs, Sylwia Handzik and Katsuhiko Ono for their excellent technical assistance.

References

Anagnostou VK, Bepler G, Syrigos KN, Tanoue L, Gettinger S, Homer RJ, Boffa D, Detterbeck F & Rimm DL 2009 High expression of mammalian target of rapamycin is associated with better outcome for patients with early stage lung adenocarcinoma. *Clinical Cancer Research* **15** 4157–4164. (doi:10.1158/1078-0432.CCR-09-0099)

Boone J, Ten Kate FJ, Offerhaus GJ, van Diest PJ, Rinkes IH & van Hillegersberg R 2008 mTOR in squamous cell carcinoma of the oesophagus: a potential target for molecular therapy? *Journal of Clinical Pathology* **61** 909–913. (doi:10.1136/jcp.2008.055772)

Campbell L, Jasani B, Edwards K, Gumbleton M & Griffiths DF 2008 Combined expression of caveolin-1 and an activated AKT/mTOR pathway predicts reduced disease-free survival in clinically confined renal cell carcinoma. *British Journal of Cancer* **98** 931–940. (doi:10.1038/sj.bjc.6604243)

Castellvi J, Garcia A, Rojo F, Ruiz-Marcellan C, Gil A, Baselga J & Ramon y Cajal S 2006 Phosphorylated 4E binding protein 1: a hallmark of cell signaling that correlates with survival in ovarian cancer. *Cancer* **107** 1801–1811. (doi:10.1002/cncr.22195)

Culjkovic B, Topisirovic I & Borden KL 2007 Controlling gene expression through RNA regulons: the role of the eukaryotic translation initiation factor eIF4E. *Cell Cycle* **6** 65–69.

Darb-Esfahani S, Faggad A, Noske A, Weichert W, Buckendahl AC, Müller B, Budczies J, Röske A, Dietel M & Denkert C 2009 Phospho-mTOR and phospho-4EBP1 in endometrial adenocarcinoma: association with stage and grade *in vivo* and link with response to rapamycin treatment *in vitro*. *Journal of Cancer Research and Clinical Oncology* **135** 933–941. (doi:10.1007/s00432-008-0529-5)

Duran I, Kortmansky J, Singh D, Hirte H, Kocha W, Goss G, Le L, Oza A, Nicklee T, Ho J *et al.* 2006 A phase II clinical and pharmacodynamic study of temsirolimus in advanced neuroendocrine carcinomas. *British Journal of Cancer* **95** 1148–1154. (doi:10.1038/sj.bjc.6603419)

Gao X, Zhang Y, Arrazola P, Hino O, Kobayashi T, Yeung RS, Ru B & Pan D 2002 Tsc tumour suppressor proteins antagonize amino-acid-TOR signalling. *Nature Cell Biology* **4** 699–704. (doi:10.1038/ncb847)

Grozinsky-Glasberg S, Franchi G, Teng M, Leontiou CA, Ribeiro de Oliveira A Jr, Dalino P, Salahuddin N, Korbonits M & Grossman AB 2008 Octreotide and the mTOR inhibitor RAD001 (everolimus) block proliferation and interact with the AKT–mTOR–p70S6K pathway in a neuro-endocrine tumour cell line. *Neuroendocrinology* **87** 168–181. (doi:10.1159/000111501)

Hay N & Sonenberg N 2004 Upstream and downstream of mTOR. *Genes and Development* **18** 1926–1945. (doi:10.1101/gad.1212704)

Iida S, Miki Y, Ono K, Akahira J, Suzuki T, Ishida K, Watanabe M & Sasano H 2010 Novel classification based

on immunohistochemistry combined with hierarchical clustering analysis in non-functioning neuroendocrine tumor patients. *Cancer Science* **101** 2278–2285. (doi:10.1111/j.1349-7006.2010.01657.x)

Kremer CL, Klein RR, Mendelson J, Browne W, Samadzedeh LK, Vanpatten K, Highstrom L, Pestano GA & Nagle RB 2006 Expression of mTOR signaling pathway markers in prostate cancer progression. *Prostate* **66** 1203–1212. (doi:10.1002/pros.20410)

Missiaglia E, Dalai I, Barbi S, Beghelli S, Falconi M, della Peruta M, Piemonti L, Capurso G, Di Florio A, delle Fave G *et al.* 2010 Pancreatic endocrine tumors: expression profiling evidences a role for AKT–mTOR pathway. *Journal of Clinical Oncology* **28** 245–255. (doi:10.1200/JCO.2008.21.5988)

Modlin IM, Lye KD & Kidd M 2003 A 5-decade analysis of 13,715 carcinoid tumors. *Cancer* **97** 934–959. (doi:10.1002/cncr.11105)

Modlin IM, Oberg K, Chung DC, Jensen RT, de Herder WW, Thakker RV, Caplin M, Delle Fave G, Kaltsas GA, Krenning EP *et al.* 2008 Gastroenteropancreatic neuroendocrine tumours. *Lancet Oncology* **9** 61–72. (doi:10.1016/S1470-2045(07)70410-2)

Moreno A, Akcakanat A, Munsell MF, Soni A, Yao JC & Meric-Bernstam F 2008 Antitumor activity of rapamycin and octreotide as single agents or in combination in neuroendocrine tumors. *Endocrine-Related Cancer* **15** 257–266. (doi:10.1677/ERC-07-0202)

Noske A, Lindenberg JL, Darb-Esfahani S, Weichert W, Buckendahl AC, Röske A, Sehouli J, Dietel M & Denkert C 2008 Activation of mTOR in a subgroup of ovarian carcinomas: correlation with p-eIF-4E and prognosis. *Oncology Reports* **20** 1409–1417. (doi:10.3892/or_0000 0160)

Oberg K & Jelic S 2008 ESMO Guidelines Working Group. Neuroendocrine gastroenteropancreatic tumors: ESMO clinical recommendations for diagnosis, treatment and follow-up. *Annals of Oncology* **19** 104–105.

Pelloski CE, Lin E, Zhang L, Yung WK, Colman H, Liu JL, Woo SY, Heimberger AB, Suki D, Prados M *et al.* 2006 Prognostic associations of activated mitogen-activated protein kinase and Akt pathways in glioblastoma. *Clinical Cancer Research* **12** 3935–3941. (doi:10.1158/1078-0432.CCR-05-2202)

Plöckinger U & Wiedenmann B 2007 Treatment of gastroenteropancreatic neuroendocrine tumors. *Virchows Archive* **451** 71–80. (doi:10.1007/s00428-007-0446-z)

Righi L, Volante M, Rapa I, Tavaglione V, Inzani F, Pelosi G & Papotti M 2010 Mammalian target of rapamycin (mTOR) signaling activation patterns in neuroendocrine tumors of the lung. *Endocrine-Related Cancer* **17** 977–987. (doi:10.1677/ERC-10-0157)

Rindi G, Klöppel G, Alhman H, Caplin M, Couvelard A, de Herder WW, Erikssson B, Falchetti A, Falconi M, Komminoth P *et al.* 2006 TNM staging of foregut (neuro)endocrine tumors: a consensus proposal including a grading system. *Virchows Archive* **449** 395–401. (doi:10.1007/s00428-006-0250-1)

Rindi G, Klöppel G, Couvelard A, Komminoth P, Körner M, Lopes JM, McNicol AM, Nilsson O, Perren A, Scarpa A *et al.* 2007 TNM staging of midgut and hindgut (neuro)endocrine tumors: a consensus proposal including a grading system. *Virchows Archive* **451** 757–762. (doi:10.1007/s00428-007-0452-1)

Rini BI 2008 Temsirolimus, an inhibitor of mammalian target of rapamycin. *Clinical Cancer Research* **14** 1286–1290. (doi:10.1158/1078-0432.CCR-07-4719)

Rojo F, Najera L, Lirola J, Jiménez J, Guzmán M, Sabadell MD, Baselga J & Ramon y Cajal S 2007 4E-binding protein 1, a cell signaling hallmark in breast cancer that correlates with pathologic grade and prognosis. *Clinical Cancer Research* **13** 81–89. (doi:10.1158/1078-0432.CCR-06-1560)

Rong L, Livingstone M, Sukarieh R, Petroulakis E, Gingras AC, Crosby K, Smith B, Polakiewicz RD, Pelletier J, Ferraiuolo MA *et al.* 2008 Control of eIF4E cellular localization by eIF4E-binding proteins, 4E-BPs. *RNA* **14** 1318–1327. (doi:10.1261/rna.950608)

Sabatini DM 2006 mTOR and cancer: insights into a complex relationship. *Nature Reviews. Cancer* **6** 729–734. (doi:10.1038/nrc1974)

Sahin F, Kannangai R, Adegbola O, Wang J, Su G & Torbenson M 2004 mTOR and P70 S6 kinase expression in primary liver neoplasms. *Clinical Cancer Research* **10** 8421–8425. (doi:10.1158/1078-0432.CCR-04-0941)

Sánchez-Fructuoso AI 2008 Everolimus: an update on the mechanism of action, pharmacokinetics and recent clinical trials. *Expert Opinion on Drug Metabolism & Toxicology* **4** 807–819. (doi:10.1517/17425255.4.6.807)

Sarbassov DD, Guertin DA, Ali SM & Sabatini DM 2005 Phosphorylation and regulation of Akt/PKB by the rictor–mTOR complex. *Science* **307** 1098–1101. (doi:10.1126/science.1106148)

Shida T, Kishimoto T, Furuya M, Nikaido T, Koda K, Takano S, Kimura F, Shimizu H, Yoshidome H, Ohtsuka M *et al.* 2010 Expression of an activated mammalian target of rapamycin (mTOR) in gastroenteropancreatic neuroendocrine tumors. *Cancer Chemotherapy and Pharmacology* **65** 889–893. (doi:10.1007/s00280-009-1094-6)

Sobin LH, Gospodarowicz MK & Wittekind C 2009 *TNM Classification of Malignant Tumours*, 7th edn, pp 86–95. Holboken, NJ: John Wiley & Sons.

Tampellini M, Longo M, Cappia S, Bacillo E, Alabiso I, Volante M, Dogliotti L & Papotti M 2007 Co-expression of EGF receptor, TGFα and S6 kinase is significantly associated with colorectal carcinomas with distant metastases at diagnosis. *Virchows Archive* **450** 321–328. (doi:10.1007/s00428-007-0370-2)

Toumpanakis CG & Caplin ME 2008 Molecular genetics of gastroenteropancreatic neuroendocrine tumors. *American Journal of Gastroenterology* **103** 729–732. (doi:10.1111/j.1572-0241.2007.01777.x)

Whalen SG, Gingras AC, Amankwa L, Mader S, Branton PE, Aebersold R & Sonenberg N 1996 Phosphorylation of eIF-4E on serine 209 by protein kinase C is inhibited by the translational repressors, 4E-binding proteins. *Journal of Biological Chemistry* **271** 11831–11837. (doi:10.1074/jbc.271.20.11831)

Yao JC, Hassan M, Phan A, Dagohoy C, Leary C, Mares JE, Abdalla EK, Fleming JB, Vauthey JN, Rashid A *et al.* 2008*a* One hundred years after "carcinoid": epidemiology of and prognostic factors for neuroendocrine tumors in 35,825 cases in the United States. *Journal of Clinical Oncology* **26** 3063–3072. (doi:10.1200/JCO.2007.15.4377)

Yao JC, Phan AT, Chang DZ, Wolff RA, Hess K, Gupta S, Jacobs C, Mares JE, Landgraf AN, Rashid A *et al.* 2008*b* Efficacy of RAD001 (everolimus) and octreotide LAR in advanced low- to intermediate-grade neuroendocrine tumors: results of a phase II study. *Journal of Clinical Oncology* **26** 4311–4318. (doi:10.1200/JCO.2008.16.7858)

Yao JC, Lombard-Bohas C, Baudin E, Kvols LK, Rougier P, Ruszniewski P, Hoosen S, St Peter J, Haas T, Lebwohl D *et al.* 2010 Daily oral everolimus activity in patients with metastatic pancreatic neuroendocrine tumors after failure of cytotoxic chemotherapy: a phase II trial. *Journal of Clinical Oncology* **28** 69–76. (doi:10.1200/JCO.2009.24.2669)

Zhou X, Tan M, Stone Hawthorne V, Klos KS, Lan KH, Yang Y, Yang W, Smith TL, Shi D & Yu D 2004 Activation of the Akt/mammalian target of rapamycin/4E-BP1 pathway by ErbB2 overexpression predicts tumor progression in breast cancers. *Clinical Cancer Research* **10** 6779–6788. (doi:10.1158/1078-0432.CCR-04-0112)

Zitzmann K, De Toni EN, Brand S, Göke B, Meinecke J, Spöttl G, Meyer HH & Auernhammer CJ 2007 The novel mTOR inhibitor RAD001 (everolimus) induces antiproliferative effects in human pancreatic neuroendocrine tumor cells. *Neuroendocrinology* **85** 54–60. (doi:10.1159/000100057)

Received in final form 11 November 2010
Accepted 13 December 2010
Made available online as an Accepted Preprint
13 December 2010

Molecular and Cellular Endocrinology 340 (2011) 168–174

Contents lists available at ScienceDirect

Molecular and Cellular Endocrinology

journal homepage: www.elsevier.com/locate/mce

Review

Suppression of estrogen actions in human lung cancer

Yasuhiro Miki[a,1], Keiko Abe[a], Satoshi Suzuki[b], Takashi Suzuki[a], Hironobu Sasano[a,*]

[a] Department of Pathology, Tohoku University Graduate School of Medicine, 2-1 Seiryo-machi, Aoba-ku, Sendai, Miyagi 980-8575, Japan
[b] Department of Thoracic Surgery, Ishinomaki Red Cross Hospital, Ishinomaki, Japan

ARTICLE INFO

Article history:
Received 23 February 2010
Received in revised form 8 February 2011
Accepted 13 February 2011

Key words:
Lung carcinoma
Estrogen receptor
Aromatase

ABSTRACT

Estrogen plays a critical role in female reproduction but has also been reported to have important roles in various target tissues expressing estrogen receptor (ER) α and/or ERβ in both male and female. ERs especially ERβ have been demonstrated to be present and functional in both normal human lung and its disorders including cancer. Non-small cell lung carcinomas (NSCLCs) are well-known to be composed of heterogeneous groups. Squamous cell carcinoma is the most common subtype in men, but adenocarcinoma is the most common histologic subtype in women. Therefore, sex steroid hormones such as estrogens have been considered to play some roles in NSCLC. In particular, results of several epidemiological analyses pointed out the association between physiological or artificial alterations of hormone status such as menstruation and postmenopausal administration of hormone replacement therapy and lung cancer risks or its development especially in female subjects. In NSCLC tissues, intratumoral estrogen synthesis via aromatase, which is a key enzyme in the estrogen synthesis involved in aromatization of androgens into estrogens, has recently become of clinical interest as a possible target of therapy. Therefore, in this review, we focused on the potential of an endocrine therapy in NSCLC using clinically available inhibitors of estrogen and aromatase actions.

Contents

1. Introduction 168
2. Estrogens and NSCLC 169
3. ERα and ERβ in NSCLC 169
4. Aromatase in NSCLC 171
5. The cross talk between estrogen and EGF signals 173
6. Conclusion 173
References 173

1. Introduction

Lung cancer is currently the most frequently diagnosed major cancer all over the world and the most common cause of cancer death in the world. Almost all lung cancers are carcinomas. Lung carcinoma is histologically classified into small cell carcinoma and non-small cell lung carcinoma (NSCLC). NSCLC is further composed of heterogeneous groups such as adenocarcinoma, squamous cell carcinoma and large cell carcinoma. Small cell carcinomas comprise about 20% of cases and large cell undifferentiated carcinomas approximately 9% (Prkin et al., 2004). In other histological types, the proportions differ by sex: squamous cell carcinomas comprise 44% of lung cancers in men but 25% in women, while adenocarcinomas comprise only 28% of the cases in men but 42% in women (Prkin et al., 2004). In adenocarcinomas, although most cases are detected in smokers, it develops more frequency than any other histologic type of lung carcinoma individuals, particularly women, among those who have never smoked (Prkin et al., 2004). Bronchioloalveolar carcinoma is a subset of lung adenocarcinoma but is associated with distinct clinical presentation, tumor biology, response to therapy, and prognosis or clinical outcome compared to other subtypes of NSCLC (Raz et al., 2006). Bronchioloalveolar carcinoma disproportionately influences women, never-smokers, and Asians and is characterized by proliferation along alveolar septa without evidence of stromal, vascular, or pleural invasion (Raz et al., 2006). It is true that more men than women developed lung cancer, and more men than women die from the disease (Payne, 2005).

* Corresponding author. Tel.: +81 22 717 8050; fax: +81 22 717 8051.
E-mail address: hsasano@patholo2.med.tohoku.ac.jp (H. Sasano).
[1] Current address: Division of Oral Pathology, Department of Oral Medicine and Surgery, Tohoku University Graduate School of Dentistry, Japan.

0303-7207/$ – see front matter © 2011 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.mce.2011.02.018