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mRNA expression of mTOR signaling components and MGMT promoter methylation status as potential predictive biomarkers in pancreatic neuroendocrine neoplasms

Medical therapeutic options for the treatment of well-differentiated pancreatic neuroendocrine neoplasms (pNENs) include the mechanistic target of rapamycin (mTOR) pathway inhibitor everolimus and the alkylating cytotoxic agent temozolomide (in combination with 5-Fluorouracil). Criteria to assess prognosis in pNEN patients are still limited, as biomarkers to predict the therapeutic response are still unknown. Several studies could show aberrant mTOR signaling pathway activity at different levels of the signaling cascade in pNENs. Moreover, the response to temozolomide correlates with the methylation-status of the promoter region of the O6-methylguanine-DNA methyltransferase (MGMT) gene in glioblastomas. In this study, we analyzed the mRNA expression of different mTOR pathway components and evaluated the *MGMT* methylation status in patients with well-differentiated pNENs in order to identify potential prognostic and predictive biomarkers (for the forementioned therapies).

In this retrospective multicenter study, real-time quantitative TaqMan reverse transcriptase-polymerase chain reaction was used to analyze mRNA expression of eleven different mTOR pathway genes (*IGF-1*, *IGFBP-3*, *PIK3CA*, *AKT-1*, *MTOR*, *MLST8*, *DEPTOR*, *RAPTOR*, *RICTOR*, *4EBP1* und *VEGF-A*), in tumor tissue of 75 patients with well-differentiated pNENs in comparison to normal pancreatic tissue. Relative gene expression was determined according to the 40-ΔCT method using *CALM2* as housekeeping gene. Additionally, we used bisulfite conversion followed by pyrosequencing to evaluate methylation status of the *MGMT* promoter in tumor tissue of 76 pNEN patients. In addition, MGMT protein expression was analyzed by immunohistochemistry in 57 patients. In a total of 70 patients mTOR gene expression, as well as MGMT promoter status were analyzed.

The analysis of mRNA expression levels of eleven genes encoding for mTOR pathway members indicated an increased signaling activity in pNENs. A distinct linear correlation only occurred between *MTOR* and *AKT-1*. Clear association between mRNA expression and a clinical feature was only found for *RAPTOR*: High *RAPTOR* mRNA expression was significantly correlated with better overall survival in unselected patients. No prediction to everolimus treatment response could be deduced by mRNA expression analysis of the mTOR pathway components in a small subgroup of 21 patients. Hypermethylation of the *MGMT* promoter region occurred in 18.4 to 63.2 % of pNENs (dependent on different cut-off values of 7 %, 10 %, 15 % and 20%). MGMT protein expression was not correlated with the methylation status. Neither MGMT protein expression, nor *MGMT* promoter methylation were associated with clinical characteristics. The response to temozolomide was not correlated with *MGMT* promoter methylation status in a small subgroup of nine patients.

Even though mRNA expression of different mTOR pathway components indicated an enhanced activity of this signaling cascade in tumor tissue of pNEN patients, we could not identify a statistical significant parameter useful for prediction of everolimus treatment response. The prognostic and predictive value of *MGMT* promoter methylation status in pNENs is discussed controversially. We could not identify an association of *MGMT* status with clinical parameters in this study. The predictive value of this study is limited due to the low number of patients who received treatment with everolimus or temozolomide. Further prospective studies analyzing the predictive value of the expression of mTOR pathway genes or the *MGMT* status are desirable.

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ORIGINAL ARTICLE

Everolimus for Advanced Pancreatic Neuroendocrine Tumors

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ABSTRACT

BACKGROUND

Everolimus, an oral inhibitor of mammalian target of rapamycin (mTOR), has shown antitumor activity in patients with advanced pancreatic neuroendocrine tumors, in two phase 2 studies. We evaluated the agent in a prospective, randomized, phase 3 study.

METHODS

We randomly assigned 410 patients who had advanced, low-grade or intermediate-grade pancreatic neuroendocrine tumors with radiologic progression within the previous 12 months to receive everolimus, at a dose of 10 mg once daily (207 patients), or placebo (203 patients), both in conjunction with best supportive care. The primary end point was progression-free survival in an intention-to-treat analysis. In the case of patients in whom radiologic progression occurred during the study, the treatment assignments could be revealed, and patients who had been randomly assigned to placebo were offered open-label everolimus.

RESULTS

The median progression-free survival was 11.0 months with everolimus as compared with 4.6 months with placebo (hazard ratio for disease progression or death from any cause with everolimus, 0.35; 95% confidence interval [CI], 0.27 to 0.45; P<0.001), representing a 65% reduction in the estimated risk of progression or death. Estimates of the proportion of patients who were alive and progression-free at 18 months were 34% (95% CI, 26 to 43) with everolimus as compared with 9% (95% CI, 4 to 16) with placebo. Drug-related adverse events were mostly grade 1 or 2 and included stomatitis (in 64% of patients in the everolimus group vs. 17% in the placebo group), rash (49% vs. 10%), diarrhea (34% vs. 10%), fatigue (31% vs. 14%), and infections (23% vs. 6%), which were primarily upper respiratory. Grade 3 or 4 events that were more frequent with everolimus than with placebo included anemia (6% vs. 0%) and hyperglycemia (5% vs. 2%). The median exposure to everolimus was longer than exposure to placebo by a factor of 2.3 (38 weeks vs. 16 weeks).

CONCLUSIONS

Everolimus, as compared with placebo, significantly prolonged progression-free survival among patients with progressive advanced pancreatic neuroendocrine tumors and was associated with a low rate of severe adverse events. (Funded by Novartis Oncology; RADIANT-3 ClinicalTrials.gov number, NCT00510068.)

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The incidence and prevalence of pancreatic neuroendocrine tumors are increasing[1-3]; these tumors represent approximately 1.3% of all cases of pancreatic cancer in incidence and 10% of cases in prevalence.[1-3] Pancreatic neuroendocrine tumors are frequently diagnosed at a late stage, with approximately 65% of patients presenting with unresectable or metastatic disease; as a result, these patients have a poor prognosis. The median survival time for patients with distant metastatic disease is 24 months,[2] and limited treatment options are available for this population.

Streptozocin is the only approved therapy for pancreatic neuroendocrine tumors in the United States; however, the role of chemotherapy in advanced cases continues to be debated.[3-12] The criteria that were used to determine the outcome measures in many earlier trials are considered unacceptable today, and a substantial number of adverse events were seen with regimens that showed improved response rates.[3,10,13,14] Large, prospective, randomized trials that use validated criteria are therefore required to show the value of promising new treatment regimens for advanced pancreatic neuroendocrine tumors. A recent prospective study (reported by Raymond et al. elsewhere in this issue of the *Journal*) shows that sunitinib has antitumor activity.[15]

Everolimus (Afinitor, Novartis Pharmaceuticals) has recently shown promising antitumor activity in two phase 2 studies involving patients with pancreatic neuroendocrine tumors.[3,16] Everolimus inhibits mammalian target of rapamycin (mTOR), a serine–threonine kinase that stimulates cell growth, proliferation, and angiogenesis.[3,16,17] Autocrine activation of the mTOR signaling pathway, mediated through insulin-like growth factor 1, has been implicated in the proliferation of pancreatic neuroendocrine tumor cells.[18] Consistent with this observation is the finding that inhibition of mTOR has a significant antiproliferative effect on pancreatic neuroendocrine tumor cell lines.[19,20]

The RAD001 in Advanced Neuroendocrine Tumors, third trial (RADIANT-3) study was conducted to determine whether everolimus, at a dose of 10 mg per day, as compared with placebo, would prolong progression-free survival among patients with advanced pancreatic neuroendocrine tumors.

METHODS

PATIENTS

Patients were eligible to be included in the study if they were 18 years of age or older and had low-grade or intermediate-grade advanced (unresectable or metastatic) pancreatic neuroendocrine tumors and radiologic documentation of disease progression (an unequivocal increase in the size of tumors) in the 12 months preceding randomization. Prior antineoplastic therapy was not an exclusion criterion. Other key eligibility criteria included the presence of measurable disease, as assessed according to the Response Evaluation Criteria in Solid Tumors (RECIST), version 1.0 (see the Supplementary Appendix, available with the full text of this article at NEJM.org)[21]; a World Health Organization (WHO) performance status of 2 or less (with 0 indicating that the patient is fully active and able to carry on all predisease activities without restriction; 1 indicating that the patient is restricted in physically strenuous activity but is ambulatory and able to carry out work of a light or sedentary nature, such as light housework or office work; and 2 indicating that the patient is ambulatory and up and about more than 50% of waking hours and is capable of all self-care but unable to carry out any work activities)[22]; adequate bone marrow, renal, and hepatic function; and adequately controlled lipid and glucose concentrations. Patients were ineligible if they had undergone hepatic-artery embolization within 6 months before enrollment (within 1 month if there were other sites of measurable disease) or cryoablation or radiofrequency ablation of hepatic metastasis within 2 months before enrollment, had any severe or uncontrolled medical conditions, had received prior therapy with an mTOR inhibitor, or were receiving long-term treatment with glucocorticoids or other immunosuppressive agents.

STUDY OVERSIGHT

The protocol was approved by the institutional review board or ethics committee at each participating center, and the study was conducted in accordance with Good Clinical Practice principles and applicable local regulations. All patients provided written informed consent.

The study was designed by the academic investigators and by representatives of the sponsor,

Novartis Oncology. The data were collected with the use of the sponsor's data management systems and were analyzed by the sponsor's statistical team. All the authors contributed to the interpretation of data and the subsequent writing, reviewing, and amending of the manuscript; the first draft of the manuscript was prepared by the first author and by a medical writer employed by Novartis Oncology. The protocol, including the statistical analysis plan, is available at NEJM.org. All the authors vouch for the accuracy and completeness of the reported data and attest that the study conformed to the protocol and statistical analysis plan.

STUDY DESIGN AND TREATMENT

In this international, multicenter, double-blind, phase 3 study, patients were randomly assigned to treatment with oral everolimus, at a dose of 10 mg once daily, or matching placebo, both in conjunction with best supportive care. Patients were stratified according to status with respect to prior chemotherapy (receipt vs. no receipt) and according to WHO performance status (0 vs. 1 or 2) at baseline.

Treatment continued until progression of the disease, development of an unacceptable toxic effect, drug interruption for 3 weeks or longer, or withdrawal of consent. The study-group assignments were concealed from the investigators, but disclosure was permitted if an investigator determined that the criteria for disease progression according to RECIST had been met and if there was an intention to switch the patient to open-label therapy. Patients who had been assigned to placebo initially could then switch to open-label everolimus. This element of the study design was incorporated to address both ethical and recruitment considerations, given that the trial involved patients with a rare disease. We recognized the potential influence of this aspect of the study design on the analysis of the end point of overall survival.

Doses were delayed or reduced if patients had clinically significant adverse events that were considered to be related to the study treatment, according to an algorithm described in the protocol. In such cases, two reductions in the dose of the study drug were permitted: an initial reduction to 5 mg daily and a subsequent reduction to 5 mg every other day.

EFFICACY AND SAFETY ASSESSMENTS

The primary end point was progression-free survival, documented by the local investigator according to RECIST and defined as the time from randomization to the first documentation of disease progression or death from any cause. If the disease had not progressed and the patient had not died as of the cutoff date for the analysis, data for progression-free survival were censored at the time of the last adequate tumor assessment — which was defined as the last assessment of overall lesion response that showed complete response, partial response, or stable disease — before the cutoff date or the date of initiation of other anticancer therapy.[23] In the primary analysis, data for progression-free survival were censored at the time of the last adequate tumor assessment if an event occurred after two or more missing tumor assessments. Data for patients without any valid post-baseline tumor assessment were censored on day 1 (the date of randomization). Secondary end points included the confirmed objective response rate (according to RECIST, version 1.0), the duration of response, overall survival, and safety.

All randomly assigned patients were assessed for efficacy (intention-to-treat analysis). Tumor measurements (assessed by triphasic computed tomography or magnetic resonance imaging) were performed at baseline and were repeated every 12 weeks. Scans were reviewed at the local site and centrally. In cases of a discrepancy between the local investigator's assessment and the radiologic assessment at the central location with respect to the determination of progression-free survival, adjudication was performed by an independent central adjudication committee comprising a board-certified radiologist and an oncologist, both of whom had extensive experience with neuroendocrine tumors. The central adjudication committee, whose members were unaware of the patients' study-group assignments and of the source of the data (local or central), selected the assessment that in their expert opinion reflected the more accurate evaluation.

All patients who received at least one dose of the study drug and had at least one follow-up assessment were evaluated for safety. Safety assessments consisted of the monitoring and recording of all adverse events, regular monitoring of hematologic and clinical biochemical levels (lab-

oratory evaluations) and vital signs, and physical examinations every 4 weeks. Adverse events were assessed according to the National Cancer Institute Common Terminology Criteria for Adverse Events, version 3.0 (http://ctep.info.nih.gov/protocolDevelopment/electronic_applications/docs/ctcaev3.pdf).

STATISTICAL ANALYSIS

The estimation of the sample size was based on the ability to detect a clinically meaningful improvement in the primary end point, which was defined as a 33% reduction in the risk of disease progression or death (a hazard ratio for progression or death of 0.67), corresponding to a 50% prolongation in median progression-free survival, from 6 months with placebo to 9 months with everolimus. We estimated that with a total of 282 progression-free survival events (i.e., disease progression or death), the study would have 92.6% power to detect a clinically meaningful improvement, with the use of an unstratified log-rank test, at a one-sided significance level of 2.5%. Taking into account the estimated rate of patient accrual and a 10% loss of the study population to follow-up, we estimated that we would have to enroll 392 patients to observe the required number of events.

Progression-free and overall survival were analyzed with the use of Kaplan–Meier methods; study groups were compared with the use of a log-rank test, stratified according to prior receipt or no prior receipt of chemotherapy and WHO performance status, and the hazard ratio was estimated with the use of a stratified Cox proportional-hazards model.

RESULTS

PATIENTS AND TREATMENT

Between July 2007 and May 2009, a total of 410 patients from 82 centers in 18 countries worldwide who had advanced pancreatic neuroendocrine tumors were randomly assigned to everolimus (207 patients) or placebo (203 patients) (see the figure in the Supplementary Appendix). The baseline demographic and clinical characteristics of the patients were well balanced between the two groups (Table 1). More than 80% of the patients had well-differentiated disease, more than 90% had metastases in the liver, and approximately 60% had received a diagnosis of pancreatic neuroendocrine tumor more than 2 years before entering the study. A total of 24% of the patients had gastrinoma, glucagonoma, VIPoma, insulinoma, or somatostatinoma. The two groups were similar with respect to prior receipt of radiother-

Table 1. Demographic and Baseline Clinical Characteristics of the Patients.

Characteristic	Everolimus (N=207)	Placebo (N=203)
Age — yr		
Median	58	57
Range	23–87	20–82
Sex — no. (%)		
Male	110 (53)	117 (58)
Female	97 (47)	86 (42)
WHO performance status — no. (%)		
0	139 (67)	133 (66)
1	62 (30)	64 (32)
2	6 (3)	6 (3)
Histologic status of tumor — no. (%)		
Well differentiated	170 (82)	171 (84)
Moderately differentiated	35 (17)	30 (15)
Unknown	2 (1)	2 (1)
Time from initial diagnosis — no. (%)		
≤6 mo	24 (12)	33 (16)
>6 mo to ≤2 yr	65 (31)	43 (21)
>2 yr to ≤5 yr	54 (26)	81 (40)
>5 yr	64 (31)	46 (23)
Time from disease progression to randomization — no. (%)		
≤1 mo	73 (35)	61 (30)
>1 mo to ≤2 mo	43 (21)	53 (26)
>2 mo to ≤3 mo	30 (14)	29 (14)
>3 mo to ≤12 mo	58 (28)	54 (27)
>12 mo	3 (1)	1 (<1)
No. of disease sites — no. of patients (%)		
1	51 (25)	62 (31)
2	85 (41)	64 (32)
≥3	70 (34)	77 (38)
Organ involved — no. (%)		
Liver	190 (92)	187 (92)
Pancreas	92 (44)	84 (41)
Lymph nodes	68 (33)	73 (36)
Lung	28 (14)	30 (15)
Bone	13 (6)	29 (14)

Table 2. Progression-free Survival.

Variable	Everolimus (N=207)	Placebo (N=203)	Difference	Hazard Ratio for Disease Progression or Death with Everolimus (95% CI)	P Value
Assessment by local investigator					
Progression-free survival events — no. (%)*	109 (53)	165 (81)			
Censored data — no. (%)	98 (47)	38 (19)			
Median progression-free survival — mo	11.0	4.6	6.4	0.35 (0.27–0.45)	<0.001
Review by central adjudication committee					
Progression-free survival events — no. (%)*	95 (46)	142 (70)			
Censored data — no. (%)	112 (54)	61 (30)			
Median progression-free survival — mo	11.4	5.4	6.0	0.34 (0.26–0.44)	<0.001

* Progression-free survival events include disease progression and death.

apy (23% of patients in the everolimus group and 20% in the placebo group), chemotherapy (50% in both groups), and somatostatin analogue therapy (49% in the everolimus group and 50% in the placebo group). Best supportive care included the use of somatostatin analogue therapy in approximately 40% of the patients.

With a median follow-up period of 17 months, the median duration of treatment with everolimus was 8.79 months (range, 0.25 to 27.47), as compared with 3.74 months (range, 0.01 to 37.79) with placebo. A total of 31% of the patients in the everolimus group, as compared with 11% in the placebo group, were administered treatment for a minimum of 12 months. The mean relative dose intensity (the ratio of administered doses to planned doses) was 0.86 in the everolimus group and 0.97 in the placebo group. Dose adjustments (reductions or temporary interruptions) were required by 59% of the patients receiving everolimus and 28% of the patients receiving placebo.

At the time the analysis was performed for this article, treatment was ongoing for 32% of the patients in the everolimus group and 13% of the patients in the placebo group; the primary reasons for discontinuation of treatment included disease progression (in 44% of patients in the everolimus group vs. 80% in the placebo group), adverse events (17% vs. 3%), withdrawal of consent (2% in both groups), and death (2% vs. 1%).

EFFICACY

The median progression-free survival (the primary end point), as assessed by the local investigators, was 11.0 months (95% confidence interval [CI], 8.4 to 13.9) in the everolimus group, as compared with 4.6 months (95% CI, 3.1 to 5.4) in the placebo group, representing a 65% reduction in the estimated risk of progression (hazard ratio for disease progression or death with everolimus, 0.35; 95% CI, 0.27 to 0.45; P<0.001) (Table 2 and Fig. 1A). The estimated proportion of patients who were alive and progression-free at 18 months was 34% (95% CI, 26 to 43) with everolimus as compared with 9% (95% CI, 4 to 16) with placebo, indicating that a sizable proportion of patients derived a prolonged benefit with everolimus.

The findings of the independent adjudicated central assessment of median progression-free survival were consistent with those of the assessment by local investigators. The median progression-free survival according to the central assessment was 11.4 months (95% CI, 10.8 to 14.8) with everolimus, as compared with 5.4 months (95% CI, 4.3 to 5.6) with placebo (hazard ratio for disease progression or death with everolimus, 0.34; 95% CI, 0.26 to 0.44; P<0.001) (Table 2 and Fig. 1B).

Prespecified subgroup analyses indicated that the benefit was maintained across subgroups. A benefit with everolimus was evident irrespective of status with respect to prior chemotherapy (receipt or no receipt), WHO performance status, age, sex, race, geographic region, status with respect to prior somatostatin analogue therapy (receipt or no receipt), and tumor grade (Fig. 1C).

Everolimus was associated with a superior response profile, as assessed according to RECIST (P<0.001 with the use of a two-sided Mann–Whit-

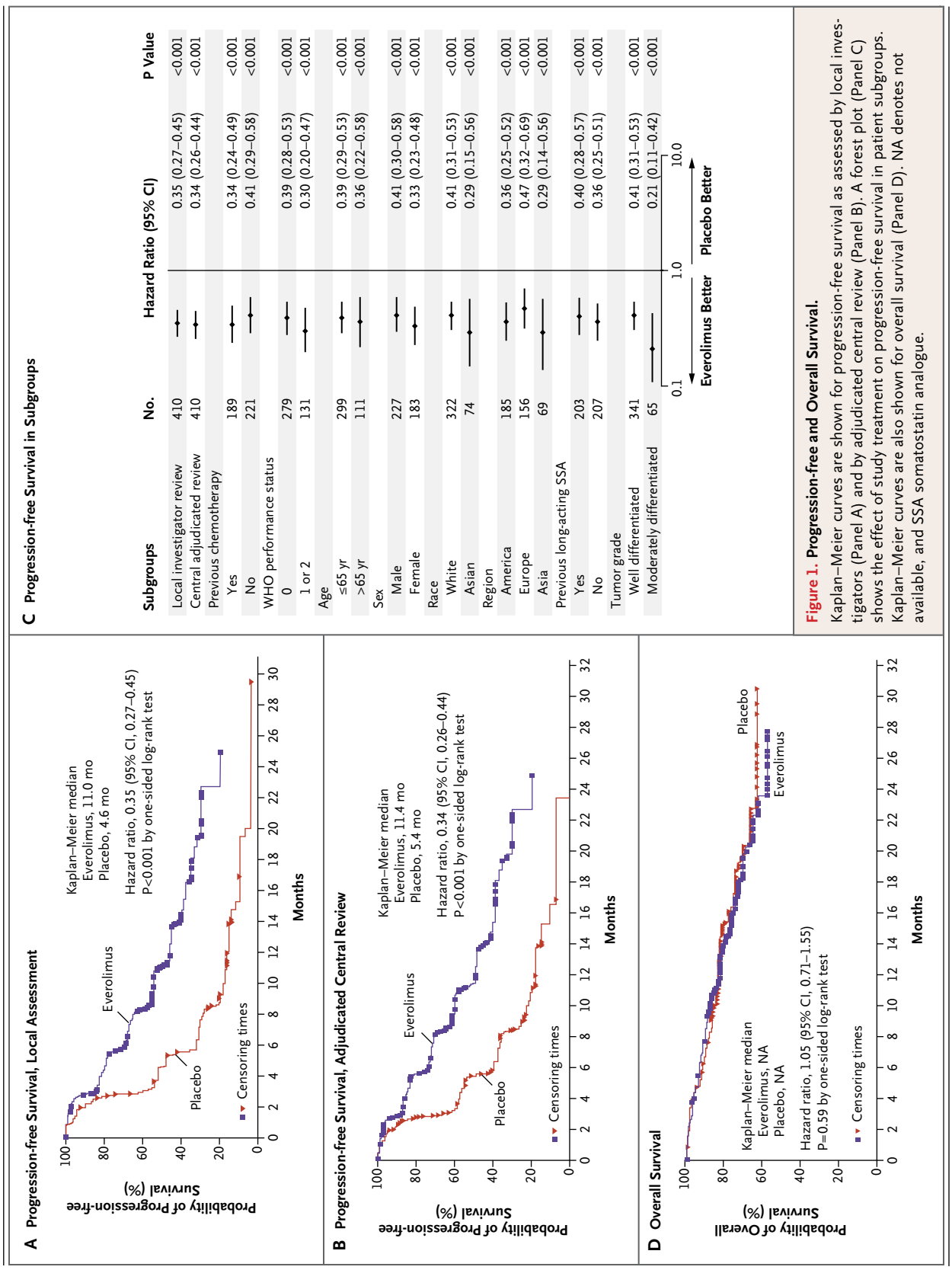
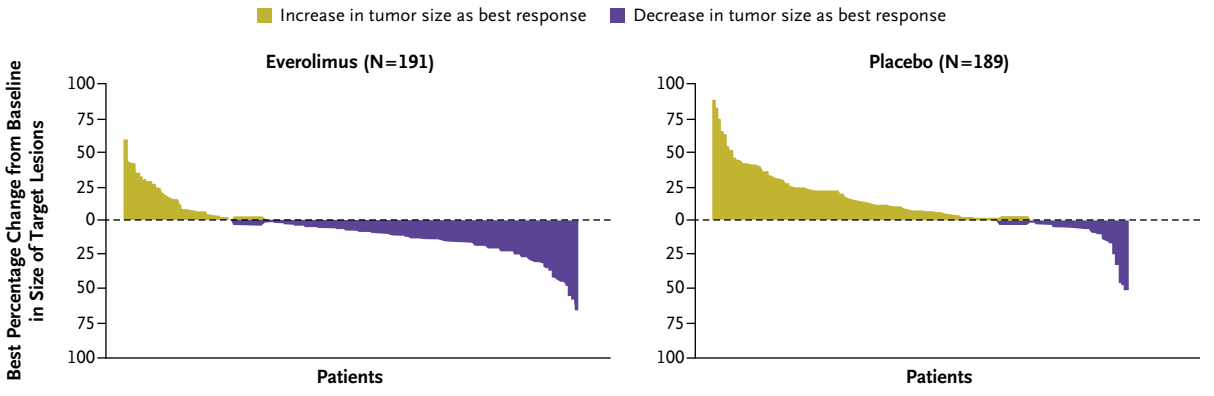


Figure 1. Progression-free and Overall Survival.

Kaplan–Meier curves are shown for progression-free survival as assessed by local investigators (Panel A) and by adjudicated central review (Panel B). A forest plot (Panel C) shows the effect of study treatment on progression-free survival in patient subgroups. Kaplan–Meier curves are also shown for overall survival (Panel D). NA denotes not available, and SSA somatostatin analogue.



	Everolimus *no. (%)*	Placebo
Decrease in size of target lesions from baseline	123 (64.4)	39 (20.6)
No change in size of target lesions from baseline	11 (5.8)	10 (5.3)
Increase in size of target lesions from baseline	43 (22.5)	112 (59.3)

Figure 2. Percentage Change from Baseline in Size of Target Lesion.

The plot shows the best percentage change from baseline in the size of the target lesion (i.e., the best response in each patient) in the everolimus group (left) and the placebo group (right). Data on 30 patients with lesions that could be evaluated in the everolimus group and 42 in the placebo group were not included in the analysis for the following reasons: 14 in the everolimus group (7.3%) and 28 in the placebo group (14.8%) showed a change in the available target lesion that contradicted the overall response of progressive disease; 1 patient in the everolimus group (0.5%) showed a change in the available target lesion, but the overall response was unknown; and the change in the target lesion could not be assessed in 15 patients in the everolimus group (7.9%) and 14 in the placebo group (7.4%).

ney U test). Confirmed objective tumor responses as assessed by local investigators (all partial responses) were observed in 10 patients receiving everolimus (5%) as compared with 4 patients receiving placebo (2%). Thus, the benefit from everolimus with respect to progression-free survival was seen primarily in the stabilization of disease or minor tumor shrinkage and in the lower incidence of progressive disease. Stable disease was evident in the case of 73% of the patients in the everolimus group as compared with 51% in the placebo group. Progressive disease as the best outcome occurred in 14% of the patients receiving everolimus and 42% of the patients receiving placebo. A total of 64% of the patients receiving everolimus, as compared with 21% receiving placebo, had some degree of tumor shrinkage (Fig. 2).

Of the 203 patients initially assigned to receive placebo, 148 (73%) crossed over to open-label everolimus, thus confounding the detection of a treatment-related survival benefit. Median overall survival was not reached at the time of this analysis, and no significant difference between the groups was observed (hazard ratio for death with everolimus, 1.05; 95% CI, 0.71 to 1.55; P=0.59) (Fig. 1D). The final analysis of overall survival will be performed once approximately 250 deaths have occurred.

SAFETY

Our findings with respect to safety were consistent with the known safety profile of everolimus, and most adverse events were grade 1 or 2. The most common drug-related adverse events occurring with a frequency of at least 10% are listed in Table 3. A total of 12 patients in the everolimus group (6%) and 4 in the placebo group (2%) died while receiving the study drug. Of these 16 deaths, 8 (5 in the everolimus group and 3 in the placebo group) were attributed to the underlying cancer or disease progression. The remaining 8 cases (7 in the everolimus group and 1 in the placebo group) were attributed to adverse events; of these, 1 in the everolimus group was related to the study drug.

The most common adverse events were stomatitis (in 64% of the patients in the everolimus group vs. 17% in the placebo group), rash (49% vs. 10%), diarrhea (34% vs. 10%), fatigue (31% vs.

14%), and infections (23% vs. 6%). Infections, as well as pneumonitis (which occurred in 12% of the patients in the everolimus group vs. 0% in the placebo group) and interstitial lung disease (2% vs. 0%), represented some of the most important clinical concerns and were primarily grade 1 or 2. The most common grade 3 or 4 drug-related adverse events were anemia, hyperglycemia, stomatitis, thrombocytopenia, diarrhea, hypophosphatemia, and neutropenia. Antibiotics were routinely prescribed for patients with infections. Glucocorticoids were administered to six of the seven patients with grade 3 or 4 noninfectious pneumonitis or interstitial lung disease; however, only 5 (2%) of these events were considered to be drug-related (Table 3). Atypical infections such as pulmonary tuberculosis, bronchopulmonary aspergillosis, and reactivation of hepatitis B (each of which occurred in one patient) were also observed in association with everolimus therapy.

The death from acute respiratory distress syndrome of one patient with insulinoma in the everolimus group (who was receiving glucocorticoid therapy) was considered to be treatment-related. Adverse events related to the study drug led to discontinuation of treatment in the case of 13% of the patients receiving everolimus (with pneumonitis, fatigue, and interstitial lung disease cited as the most common reasons) and 2% of the patients in the placebo group (as a result of cardiac failure, diarrhea, confusion and depressed level of consciousness, and elevated alanine aminotransferase concentrations). The most common drug-related adverse events necessitating dose adjustment were stomatitis (in 10% of the patients in the everolimus group vs. <1% in the placebo group), pneumonitis (7% vs. 0%), thrombocytopenia (7% vs. 0%), diarrhea (4% vs. 0%), and anemia (3% vs. 0%).

Table 3. Drug-Related Adverse Events Occurring in at Least 10% of Patients.

Adverse Event	Everolimus (N=204)		Placebo (N=203)	
	All Grades	Grade 3 or 4	All Grades	Grade 3 or 4
	no. of patients (%)			
Stomatitis*	131 (64)	14 (7)	34 (17)	0
Rash	99 (49)	1 (<1)	21 (10)	0
Diarrhea	69 (34)	7 (3)	20 (10)	0
Fatigue	64 (31)	5 (2)	29 (14)	1 (<1)
Infections†	46 (23)	5 (2)	12 (6)	1 (<1)
Nausea	41 (20)	5 (2)	37 (18)	0
Peripheral edema	41 (20)	1 (<1)	7 (3)	0
Decreased appetite	40 (20)	0	14 (7)	2 (1)
Headache	39 (19)	0	13 (6)	0
Dysgeusia	35 (17)	0	8 (4)	0
Anemia	35 (17)	12 (6)	6 (3)	0
Epistaxis	35 (17)	0	0	0
Pneumonitis‡	35 (17)	5 (2)	0	0
Weight loss	32 (16)	0	9 (4)	0
Vomiting	31 (15)	0	13 (6)	0
Pruritus	30 (15)	0	18 (9)	0
Hyperglycemia	27 (13)	11 (5)	9 (4)	4 (2)
Thrombocytopenia	27 (13)	8 (4)	1 (<1)	0
Asthenia	26 (13)	2 (1)	17 (8)	2 (1)
Nail disorder	24 (12)	1 (<1)	2 (1)	0
Cough	22 (11)	0	4 (2)	0
Pyrexia	22 (11)	0	0	0
Dry skin	21 (10)	0	9 (4)	0

* Included in this category are stomatitis, aphthous stomatitis, mouth ulceration, and tongue ulceration.
† All types of infections are included.
‡ Included in this category are pneumonitis, interstitial lung disease, lung infiltration, and pulmonary fibrosis.

DISCUSSION

In this trial, we compared everolimus with placebo in patients with advanced pancreatic neuroendocrine tumors in whom the disease had progressed within the previous 12 months. The majority of patients had received prior treatment with chemotherapy, radiotherapy, somatostatin analogue therapy, or some combination of those therapies. Everolimus, as compared with placebo, was associated with a 6.4-month prolongation of the median progression-free survival (an increase by a factor of 2.4). The patients in our study, who otherwise had a poor prognosis, had a 65% reduction in the relative risk of progression with everolimus therapy as compared with placebo ($P<0.001$). This study confirmed the prolonged progression-free survival that had been observed with everolimus in earlier phase 2 studies.[3,16]

Although the molecular pathogenesis of sporadic pancreatic neuroendocrine tumors is unknown, several genetic cancer syndromes involving the mTOR pathway, including tuberous sclerosis, neurofibromatosis, and von Hippel–Lindau disease, are linked to the development of pancreatic

neuroendocrine tumors.[24] In sporadic pancreatic neuroendocrine tumors, down-regulation of tuberin (TSC2) and phosphatase and tensin homologue (PTEN) leads to dysregulation of the mTOR pathway. Low TSC2 and PTEN are linked to progression of the cancer, an increased rate of proliferation (as assessed by Ki 67 labeling), and shortened progression-free and overall survival.[20] In a study of paired biopsy specimens, treatment with everolimus reduced tumor proliferation in neuroendocrine tumors, as evidenced by a decreasing percentage of cells with Ki 67 labeling.[16] The magnitude of the clinical benefit observed in our study confirms the importance of the mTOR pathway in pancreatic neuroendocrine tumors.

Sunitinib, an oral inhibitor of a number of tyrosine kinases (but not an inhibitor of mTOR), also shows activity against advanced pancreatic neuroendocrine tumors.[15] It is not yet clear whether sunitinib and everolimus can be combined and, if so, whether antitumor activity would be further increased with combined treatment.

We have previously shown that everolimus can be safely administered to patients with neuroendocrine tumors either with or without concurrent octreotide long-acting release (LAR) therapy.[3] The safety profile of everolimus in the current study was consistent with that in previous phase 2 studies. Despite a significantly longer duration of exposure in the population of patients with pancreatic neuroendocrine tumors, the rate of adverse events was similar to that in phase 3 trials involving patients with renal-cell carcinoma.[25] The most common drug-related adverse event in our trial was stomatitis or aphthous ulceration, characterized by sporadic occurrences of discrete white ulcerations that frequently appeared and resolved during treatment. Everolimus therapy can also be associated with mild lymphopenia and neutropenia. Although in our trial, infections were more common among patients receiving everolimus than among those receiving placebo, grade 3 or 4 drug-related infections occurred in only 2% of the patients in the everolimus group. The most commonly reported infections were mild upper respiratory infections. Adverse events were generally manageable, as evidenced by the low rate of discontinuation of treatment. Noninfectious pneumonitis and interstitial lung disease, potentially serious adverse events associated with sirolimus (previously called rapamycin) derivatives, were also observed, but these events can be effectively managed according to existing treatment guidelines.

In summary, our study shows that everolimus, as compared with placebo, improves progression-free survival in patients with advanced pancreatic neuroendocrine tumors. The adverse events seen with everolimus were mainly grade 1 and 2 events, thus allowing for long-term daily administration.

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Disclosure forms provided by the authors are available with the full text of this article at NEJM.org.

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Everolimus plus octreotide long-acting repeatable for the treatment of advanced neuroendocrine tumours associated with carcinoid syndrome (RADIANT-2): a randomised, placebo-controlled, phase 3 study

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Summary

Background **Everolimus, an oral inhibitor of the mammalian target of rapamycin (mTOR), has shown antitumour activity in patients with advanced pancreatic neuroendocrine tumours. We aimed to assess the combination of everolimus plus octreotide long-acting repeatable (LAR) in patients with low-grade or intermediate-grade neuroendocrine tumours (carcinoid).**

Methods **We did a randomised, double-blind, placebo-controlled, phase 3 study comparing 10 mg per day oral everolimus with placebo, both in conjunction with 30 mg intramuscular octreotide LAR every 28 days. Randomisation was by interactive voice response systems. Participants were aged 18 years or older, with low-grade or intermediate-grade advanced (unresectable locally advanced or distant metastatic) neuroendocrine tumours, and disease progression established by radiological assessment within the past 12 months. Our primary endpoint was progression-free survival. Adjusted for two interim analyses, the prespecified boundary at final analysis was p≤0·0246. This study is registered at ClinicalTrials.gov, number NCT00412061.**

Findings **429 individuals were randomly assigned to study groups; 357 participants discontinued study treatment and one was lost to follow-up. Median progression-free survival by central review was 16·4 (95% CI 13·7–21·2) months in the everolimus plus octreotide LAR group and 11·3 (8·4–14·6) months in the placebo plus octreotide LAR group (hazard ratio 0·77, 95% CI 0·59–1·00; one-sided log-rank test p=0·026). Drug-related adverse events (everolimus plus octreotide LAR *vs* placebo plus octreotide LAR) were mostly grade 1 or 2, and adverse events of all grades included stomatitis (62% *vs* 14%), rash (37% *vs* 12%), fatigue (31% *vs* 23%), and diarrhoea (27% *vs* 16%).**

Interpretation **Everolimus plus octreotide LAR, compared with placebo plus octreotide LAR, improved progression-free survival in patients with advanced neuroendocrine tumours associated with carcinoid syndrome.**

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Introduction

Neuroendocrine tumours, also known as carcinoids, are uncommon tumours arising from various primary sites.[1] Nearly 50% of patients with neuroendocrine tumours have metastatic disease, and 65% will die within 5 years of diagnosis.[1] The 5 year survival rate for patients with advanced neuroendocrine tumours is greater for patients with well differentiated (low or intermediate grade) versus poorly differentiated tumours and locoregional versus distant disease.[1] Survival also varies by primary site; in patients with low-grade or intermediate-grade histology and distant disease, lung and colon are associated with the worst median survival (17 and 7 months, respectively), and jejunum, ileum, and caecum are associated with the best (55–65 months).[1]

Somatostatin analogues, such as octreotide and lanreotide, improve the hormone-related symptoms associated with neuroendocrine tumours. Furthermore, octreotide long-acting repeatable (LAR) has also shown antitumour activity, prolonging time to disease progression in patients with midgut neuroendocrine tumours.[2,3] No approved antitumour drugs are available for treating progressive disease in patients with gastrointestinal or lung neuroendocrine tumours.

Overactivation of the mammalian target of rapamycin (mTOR), a serine-threonine kinase that regulates cell growth, proliferation, metabolism, and angiogenesis, has been implicated in the pathogenesis of neuroendocrine tumours.[4–7] Autocrine activation of the mTOR signalling pathway, mediated through insulin-like growth factor I, has been associated with neuroendocrine tumour cell proliferation,[8] and inhibition of the mTOR pathway has shown antiproliferative effects in cell lines of neuroendocrine tumours[9,10] and primary cultures of human neuroendocrine tumours.[11] Everolimus, an oral inhibitor of mTOR, showed promising antitumour activity in advanced neuroendocrine tumours in two phase 2 studies.[12,13] Recently, everolimus showed a 6·4 month increase in progression-free survival compared with placebo in patients with advanced pancreatic

neuroendocrine tumours.[14] However, the role of everolimus in neuroendocrine tumours of other primary sites or in combination with other drugs has not been studied extensively. Combination therapy with everolimus plus octreotide LAR might enhance antitumour efficacy by simultaneously targeting upstream and downstream components of the mTOR pathway (webappendix p 1).[15,16]

See Online for webappendix

We aimed to establish whether 10 mg per day everolimus plus 30 mg octreotide LAR every 28 days compared with placebo plus 30 mg octreotide LAR every 28 days prolongs progression-free survival in patients with well differentiated or moderately differentiated advanced neuroendocrine tumours (carcinoid tumours) and a history of flushing, diarrhoea, or both.

Methods

Participants

Between Jan 10, 2007, and April 2, 2010, we did a multicentre, double-blind, phase 3 study in Australia, Belgium, Canada, Czech Republic, Finland, France, Germany, Greece, Israel, Italy, Netherlands, Slovakia, Spain, Sweden, Turkey, and the USA. We judged the participants eligible if they were aged 18 years or older, had low-grade or intermediate-grade advanced (unresectable locally advanced or distant metastatic) neuroendocrine tumours, and disease progression established by radiological assessment within the past 12 months. Our other key eligibility criteria were history of secretory symptoms (diarrhoea or flushing) attributable to carcinoid syndrome; presence of measurable disease according to Response Evaluation Criteria In Solid Tumors version 1.0 (RECIST; webappendix pp 39–40 [amended protocol pp 38–39]);[17] WHO performance status of 2 or less;[18] adequate bone marrow, renal, and hepatic function; and adequately controlled lipid concentrations. Patients were ineligible if they had poorly differentiated or high-grade neuroendocrine carcinomas.

All participants provided written informed consent. Our protocol was approved by the institutional review board or ethics committee at each participating centre. Our study was done in accordance with Good Clinical Practice and the Declaration of Helsinki. Our study was monitored by an independent data monitoring committee and overseen by the protocol steering committee.

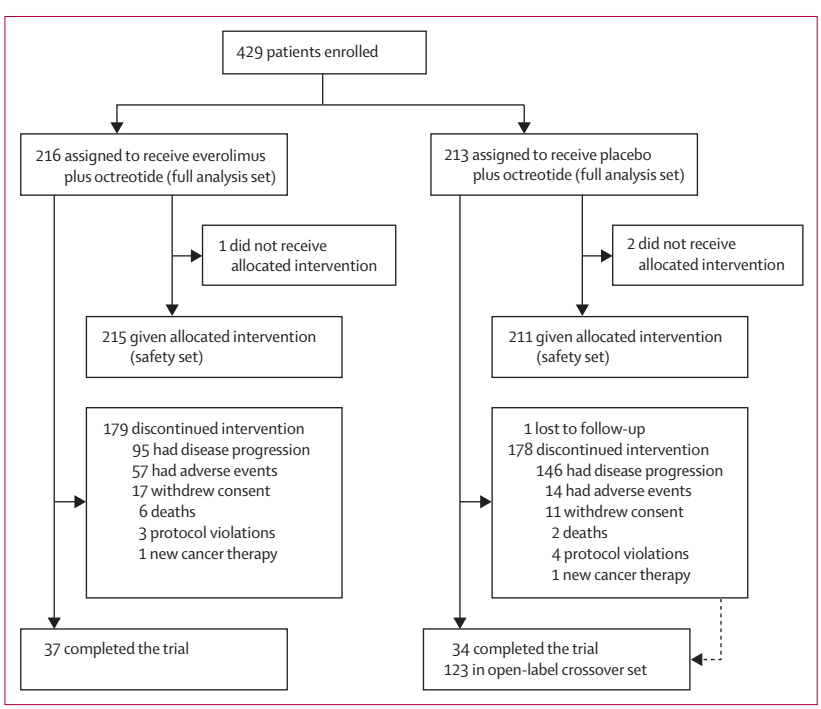


Figure 1: **Trial profile**

Randomisation and masking

Randomisation was by interactive voice response systems. Study group assignments were masked from participants and investigators, but disclosure was permitted in cases of investigator-documented disease progression according to RECIST. Participants assigned to placebo plus octreotide LAR could cross over to open-label everolimus plus octreotide LAR after disease progression was established by the investigator.

Procedures

We randomly assigned participants (1:1) to receive treatment with 10 mg oral everolimus once daily or matching placebo, both in conjunction with intramuscular 30 mg octreotide LAR every 28 days. Treatment continued until disease progression, withdrawal from treatment because of adverse events, or withdrawal of consent. Dose adjustments were permitted for safety (webappendix pp 43–44 [amended protocol pp 42–43]).

Our primary endpoint was progression-free survival according to RECIST, defined as time from random assignment to first recorded disease progression or death from any cause. Progression-free survival for our primary analysis was established by an adjudicated central review. Adjudication was done by an independent committee—from which treatment allocation was masked—assessing any discrepancies in event type or timing between local and central radiology review. Investigator-assessed progression-free survival was done as a key supportive analysis. Our secondary endpoints were objective response rate (according to RECIST), overall survival, changes from baseline in 5-hydroxyindoleacetic acid and chromogranin A concentrations, and safety.

We assessed efficacy in our full analysis set, composed of all patients randomly assigned to a study group. Tumour measurements (assessed by multiphasic CT or MRI) were done at baseline and repeated every 12 weeks.

We collected serum chromogranin A and 24 h urine samples for 5-hydroxyindoleacetic acid at baseline and, if raised (greater than the upper limit of normal) we repeated the collection on day 1 of each subsequent cycle (webappendix p 248).

In our safety population we included all patients who received at least one dose of study drug and had at least one post-baseline safety assessment. Safety assessments included monitoring of adverse events, vital signs, physical examinations every 4 weeks, chest radiograph every 12 weeks, and regular monitoring of haematological and clinical biochemistry values (laboratory assessments). We classified adverse events in accordance with the National Cancer Institute's Common Terminology Criteria for Adverse Events version 3.0.

Statistical analysis

We based our estimates of sample size on the ability to detect a clinically meaningful prolongation of progression-free survival, which we defined as a 33% reduction in the risk for disease progression or death (hazard ratio [HR] for progression or death 0·67), corresponding to a prolongation in median progression-free survival from 9 months with placebo plus octreotide LAR to 13·5 months with everolimus plus octreotide LAR. With a uniform accrual of 29 patients per month over 60 weeks and a minimum follow-up of 90 weeks, we needed 350 patients to obtain 287 progression-free survival events, which would yield 92·2% power with the use of an unstratified log-rank test at a one-sided significance level of 2·5%. With an estimated 10% of patients lost to follow-up, we targeted a total sample size of 390 patients. However, because of a loss of central radiology progression-free survival events (informative censoring), our study was amended to end on a date that allowed for a minimum follow-up of about 2 years in randomly assigned patients (April 2, 2010) irrespective of the available number of events. Adjusted for two interim analyses and the final number of progression-free survival events recorded, the significance boundary on the p-value scale at final analysis was 0·0246.

We assessed progression-free and overall survival with Kaplan-Meier methods and we compared study groups with log-rank tests. We calculated HRs and corresponding CIs with a Cox proportional hazards model. We used a prespecified marginal structural Cox proportional hazards model with the inverse probability of censoring weights (IPCW) method to assess for potential bias related to informative censoring (webappendix pp 249–251). We defined chromogranin A and 5-hydroxyindoleacetic acid responses as normalisation or a 50% or greater reduction from baseline. We described responses by treatment group, and we assessed changes from baseline over time with a mixed-effects model, including treatment, time, and the interaction term between time and treatment as fixed effects, baseline measurements as covariates, and patient as random effect. The protocol, including the statistical analysis plan, is available in the webappendix (pp 2–247). This study is registered at ClinicalTrials.gov, number NCT00412061.

Role of the funding source

The study was designed by the academic investigators and by representatives of the sponsor. Data were collected with the use of the sponsor's data management systems

For the **Common Terminology Criteria for Adverse Events** see http://ctep.cancer.gov/protocolDevelopment/electronic_applications/docs/ctcaev3.pdf

	Everolimus plus octreotide LAR group (n=216)	Placebo plus octreotide LAR group (n=213)
Median age, years (range)	60 (22–83)	60 (27–81)
Number of women	119 (55%)	89 (42%)
Number of men	97 (45%)	124 (58%)
WHO performance status*		
0	118 (55%)	140 (66%)
1	84 (39%)	62 (29%)
2	14 (6%)	10 (5%)
Primary site of cancer		
Small intestine	111 (51%)	113 (53%)
Lung	33 (15%)	11 (5%)
Colon	14 (6%)	14 (7%)
Pancreas	11 (5%)	15 (7%)
Liver	7 (3%)	11 (5%)
Other	40 (19%)	48 (23%)
Missing	0	1 (0·5%)
Histological grade		
Well differentiated	166 (77%)	175 (82%)
Moderately differentiated	38 (18%)	30 (14%)
Poorly differentiated	1 (0·5%)	1 (0·5%)
Unknown	11 (5%)	6 (3%)
Missing	0	1 (0·5%)
Current tumour-related symptoms†	170 (79%)	172 (81%)
Organ type involved‡		
Liver	198 (92%)	196 (92%)
Lymph nodes	80 (37%)	85 (40%)
Lung	64 (30%)	52 (24%)
Bone	35 (16%)	24 (11%)
Other	103 (48%)	103 (48%)
Time since initial diagnosis		
≤6 months	15 (7%)	23 (11%)
>6 months to ≤2 years	45 (21%)	53 (25%)
>2 years to ≤5 years	68 (31%)	51 (24%)
>5 years to ≤10 years	60 (28%)	61 (29%)
>10 years	27 (13%)	23 (11%)
Missing	1 (0·5%)	2 (1%)
History of previous somatostatin analogue therapy	173 (80%)	166 (78%)
History of previous octreotide therapy	169 (78%)	152 (71%)
Mean duration of previous somatostatin analogue exposure, years (SD; range)	2·6 (2·49; 0·0–11·7)	2·6 (2·39; 0·0–12·5)
Other systemic antitumour drugs	99 (46%)	82 (38%)
Chemotherapy	75 (35%)	55 (26%)
Immunotherapy	27 (13%)	20 (9%)
Targeted therapy	15 (7%)	16 (8%)
Other	21 (10%)	28 (13%)

Data are n (%) unless otherwise stated. *Data missing for one patient in the placebo plus octreotide LAR group. †Defined as diarrhoea, flushing, or both. ‡Organs as per target and non-target lesion locations recorded at baseline by investigator.

Table 1: **Baseline demographics and disease characteristics**

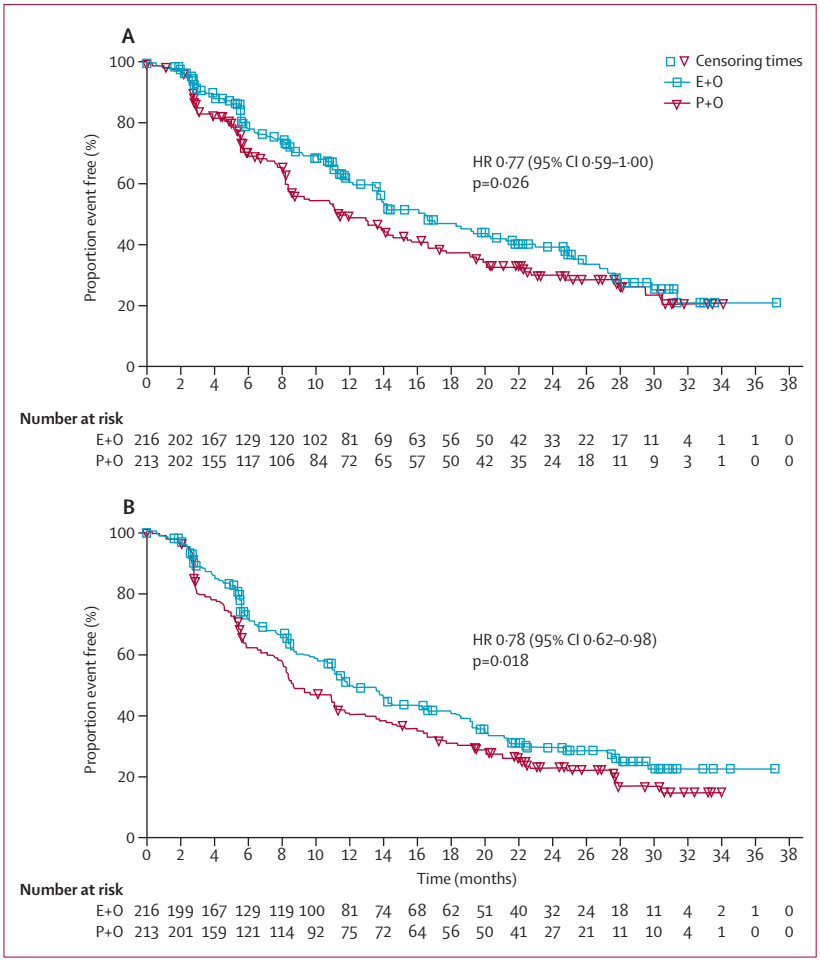


Figure 2: **Kaplan-Meier plots of progression-free events**
Assessed by central radiology review (A) and local investigator review (B). E+O=everolimus plus octreotide LAR. P+O=placebo plus octreotide LAR. HR=hazard ratio.

and were analysed by the sponsor's statistical team. All authors contributed to the interpretation of data and subsequent writing, reviewing, and amending of the report; the first draft of the report was prepared by the first author, the corresponding author, and a medical writer funded by Novartis. All authors vouch for the accuracy and completeness of the reported data and attest that the study conformed to the protocol and statistical analysis plan.

Results

Figure 1 shows the trial profile. 211 patients (98%) assigned to receive everolimus plus octreotide LAR and 204 (96%) assigned to receive placebo plus octreotide LAR had metastatic disease. There were imbalances in baseline demographic and disease characteristics favouring placebo plus octreotide LAR, including lung as primary tumour site, WHO performance status greater than 0, and previous use of chemotherapy (table 1). Both groups were similar with respect to history of previous treatment with somatostatin analogues given in accordance with site standard of care.

With a median follow-up of 28 months, the median duration of treatment was 37·0 weeks (range 1–163) in the everolimus plus octreotide LAR group and 36·6 (<1–152) in the placebo plus octreotide LAR group. Mean relative dose intensity (ratio of administered to planned doses) was 0·83 in the everolimus plus octreotide LAR group and 0·97 in the placebo plus octreotide LAR group. Dose reductions or temporary interruptions were needed by 140 patients (65%) in the everolimus plus octreotide LAR group and 74 (35%) in the placebo plus octreotide LAR group. At data cutoff, roughly equal proportions of patients in both groups remained on treatment; the primary reason for treatment discontinuation was disease progression (figure 1).

Median progression-free survival assessed by central review and based on 103 events in the everolimus plus octreotide LAR group and 120 in the placebo plus octreotide LAR group was 16·4 months (95% CI 13·7–21·2) in the everolimus plus octreotide LAR group and 11·3 (8·4–14·6) in the placebo plus octreotide LAR group. Everolimus plus octreotide LAR was associated with a 23% reduction in the estimated risk for progression (figure 2). Findings of the local investigator assessment, based on 128 events in the everolimus plus octreotide LAR group and 156 in the placebo plus octreotide LAR group), were consistent with the central review: 12·0 months (10·6–16·1) in the everolimus plus octreotide LAR group and 8·6 (8·1–11·1) in the placebo plus octreotide LAR group (figure 2). IPCW analysis confirmed the presence of informative censoring in the central assessment (treatment effect HR 0·60, 95% CI 0·44–0·84). Prespecified subgroup analyses showed a consistent benefit across most subgroups of patients. Treatment benefit with everolimus plus octreotide LAR was recorded irrespective of having or not having received previous chemotherapy and irrespective of WHO performance status, age, sex, tumour grade, and primary tumour site (figure 3). We also noted a benefit for everolimus plus octreotide LAR in the 47 patients in the everolimus plus octreotide LAR group and 61 in the placebo plus octreotide LAR group who had not been treated with octreotide LAR before study entry (median progression-free survival 25·2 months in the everolimus plus octreotide LAR group *vs* 11·3 in the placebo plus octreotide LAR group; HR 0·61, 95% CI 0·36–1·04). This might be attributable to a more substantial inhibition of the phosphoinositide 3-kinase/Akt/mTOR pathway, with everolimus and octreotide LAR inhibiting mTOR and the upstream insulin-like growth factor I autocrine loop, respectively.[15,16]

The combination of everolimus plus octreotide LAR offered patients with progressive advanced disease a 23% reduction in the relative risk of progression (HR 0·77; p=0·026). These findings were strongly supported by the local investigator-assessed analysis of progression-free

survival (HR 0·78; p=0·018) and IPCW analysis. Most adverse events associated with everolimus plus octreotide LAR were grade 1 or 2 and consistent with the known safety profile of these drugs.

Partial response as best overall response, assessed by central radiology review, was recorded in five patients in the everolimus plus octreotide LAR group and four patients in the placebo plus octreotide LAR group. Stable disease (best overall response) was evident in 182 patients (84%) in the everolimus plus octreotide LAR group and 172 (81%) in the placebo plus octreotide LAR group. Progressive disease was recorded in nine patients (4%) in the everolimus plus octreotide LAR group and 26 (12%) in the placebo plus octreotide LAR group. Of patients that could be assessed, 150 (75%) in the everolimus plus octreotide LAR group and 91 (45%) in the placebo plus octreotide LAR group experienced tumour shrinkage (figure 4).

Patients treated with everolimus plus octreotide LAR had higher proportions of chromogranin A and 5-hydroxyindoleacetic acid responses (75 [46%] of 164 and 85 [61%] of 140) compared with those treated with placebo plus octreotide LAR (53 [36%] of 146 and 76 [54%] of 141). Based on the mixed model, everolimus plus octreotide LAR resulted in greater reductions in serum chromogranin A (p treatment=0·0041) and urine 5-hydroxyindoleacetic acid (p treatment <0·0001) compared with placebo plus octreotide LAR (figure 5).

At disease progression, patients initially randomly assigned to receive placebo plus octreotide LAR were given the opportunity to cross over to open-label everolimus plus octreotide LAR, thus confounding a possible treatment-related survival benefit. 124 of the 213 patients initially assigned to receive placebo plus octreotide LAR crossed over. Of these patients, 123 (58%) also had an open-label safety assessment. Median overall survival was not reached at the time of our analysis, and we noted no significant difference between groups (HR 1·22, 95% CI 0·91–1·62). Adjusted for imbalances in baseline prognostic factors, the HR was 1·06 (0·79–1·43) (prespecified baseline covariates were age, sex, race, performance status, and previous somatostatin analogue use).

Most adverse events associated with everolimus plus octreotide LAR were grade 1 or 2 and consistent with the known safety profiles of these drugs (table 2). 18 patients in the everolimus plus octreotide LAR group and 11 in the placebo plus octreotide LAR group died within 28 days of the last intake of study drug. Of these deaths, six in the everolimus plus octreotide LAR group and six in the placebo plus octreotide LAR group were attributable to underlying cancer or disease progression. None of the remaining deaths (12 in the everolimus plus octreotide LAR group and five in the placebo plus octreotide LAR group) were deemed treatment related per investigator assessment. Drug-related adverse events led to study discontinuation in 40 patients (19%) in the everolimus plus octreotide LAR group and seven (3%) in the placebo plus octreotide LAR group.

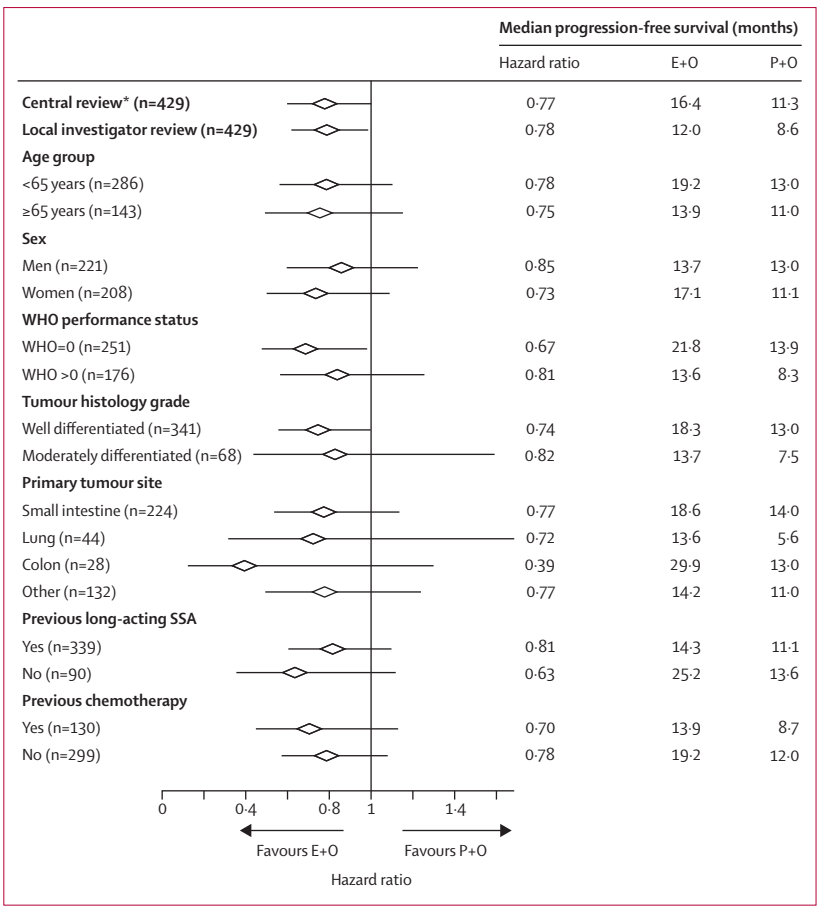
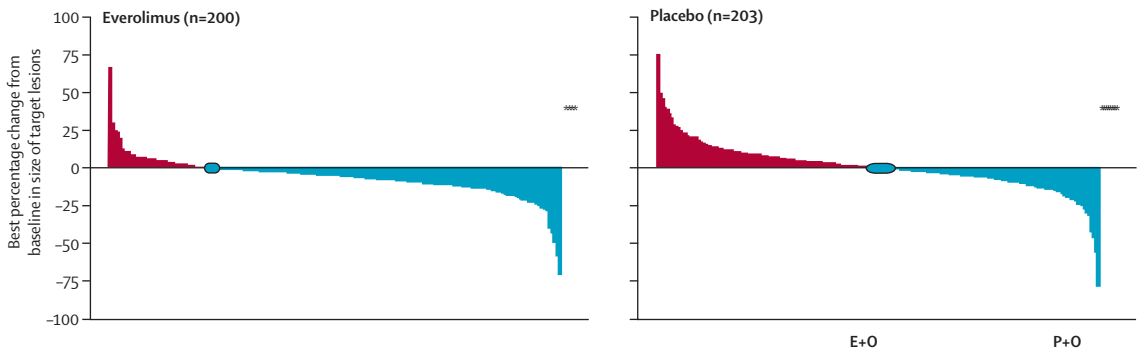


Figure 3: **Effect of study treatment on progression-free survival by subgroup**
Hazard ratio is everolimus plus octreotide LAR over placebo plus octreotide LAR, obtained by unstratified Cox model. E+O=everolimus plus octreotide LAR. P+O=placebo plus octreotide LAR. SSA=somatostatin analogue. *Independent adjudicated central review.

The most common drug-related adverse events of any grade were stomatitis, rash, fatigue, and diarrhoea (table 2). The most common grade 3 or 4 drug-related adverse events were stomatitis, fatigue, diarrhoea, hyperglycaemia, thrombocytopenia, and infections. The incidence of drug-related pneumonitis, a known issue with everolimus treatment, was 8% (18 patients) in the everolimus plus octreotide LAR group versus 0% in the placebo plus octreotide LAR group. Metabolic-related adverse events (drug related) included hyperglycaemia (table 2) and hypercholesterolaemia (12 patients [6%] in the everolimus plus octreotide LAR group *vs* three [1%] in the placebo plus octreotide LAR group). Serious adverse events were reported in 122 patients (57%) in the everolimus plus octreotide LAR group versus 73 (35%) in the placebo plus octreotide LAR group, and, of these patients, 41 (19%) versus nine (4%) reported treatment-related effects. The most commonly reported drug-related serious adverse events included diarrhoea (four patients



	E+O	P+O
Increase in size of target lesions from baseline	43 (22%)	94 (46%)
No change in size of target lesions from baseline	3 (2%)	10 (5%)
Decrease in size of target lesions from baseline	150 (75%)	91 (45%)
Change in size of target lesion was available but contradicted by overall lesion response of progressive disease	4 (2%)	8 (4%)

Figure 4: **Best percentage change from baseline in size of target lesion**
We did not include data on 16 patients in the everolimus plus octreotide group and 10 in the placebo plus octreotide LAR group in our analysis because one patient in the everolimus group showed a change in the available target lesion, although the overall response was unknown, and because change in the target lesion could not be assessed in 15 patients in the everolimus plus octreotide LAR group and 10 in the placebo plus octreotide LAR group. Additionally, four patients in the everolimus plus octreotide LAR group (2%) and eight in the placebo plus octreotide LAR group (4%) showed changes in the available target lesion contradicted by progressive disease as overall response (marked as * in the graph). E+O=everolimus plus octreotide LAR. P+O=placebo plus octreotide LAR.

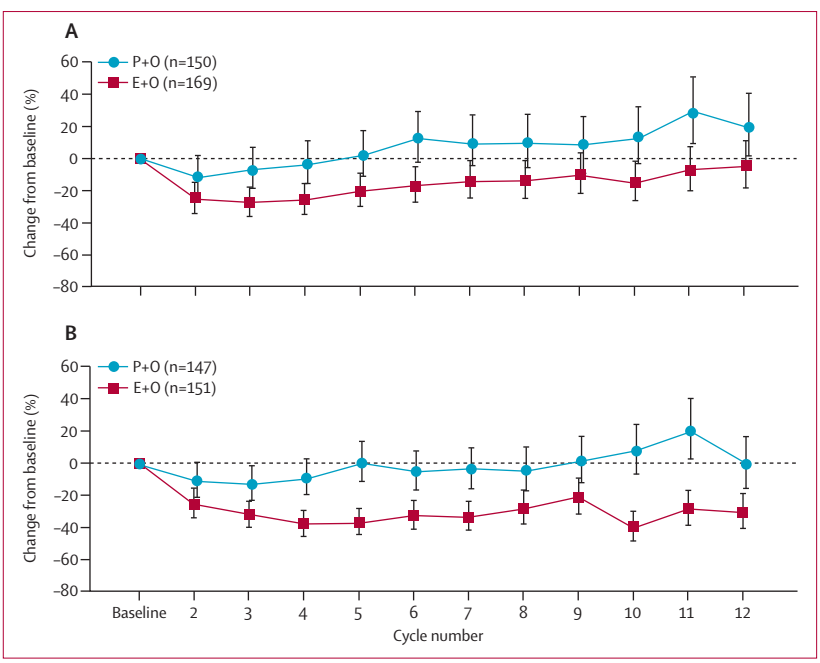


Figure 5: **Changes in biomarker concentrations over time by treatment group**
Least square estimated fold changes over baseline and associated 95% CIs derived from a mixed model are shown for serum chromogranin A (A) and 24 h urinary 5-hydroxyindoleacetic acid concentrations (B). We include only patients with raised biomarker concentrations (ie, greater than the upper limit of normal) at baseline. E+O=everolimus plus octreotide LAR. P+O=placebo plus octreotide LAR.

[2%] *vs* one [1%]), interstitial lung disease (three [1%] *vs* none), and thrombocytopenia (three [1%] *vs* none). The most commonly reported adverse events leading to discontinuation of treatment with everolimus plus octreotide LAR were fatigue (five patients; 2%), diarrhoea (four; 2%), general physical health deterioration (four; 2%), interstitial lung disease (four; 2%), and pneumonia (four; 2%).

	Everolimus plus octreotide LAR group (n=215)		Placebo plus octreotide LAR group (n=211)	
	All grades	Grades 3 and 4	All grades	Grades 3 and 4
Stomatitis*	133 (62%)	14 (7%)	29 (14%)	0
Rash	80 (37%)	2 (1%)	26 (12%)	0
Fatigue	67 (31%)	14 (7%)	49 (23%)	6 (3%)
Diarrhoea	59 (27%)	13 (6%)	33 (16%)	5 (2%)
Nausea	42 (20%)	1 (0·5%)	34 (16%)	2 (1%)
Infections†	42 (20%)	11 (5%)	13 (6%)	1 (0·5%)
Dysgeusia	36 (17%)	1 (0·5%)	7 (3%)	0
Anaemia	33 (15%)	3 (1%)	10 (5%)	0
Decreased weight	32 (15%)	1 (0·5%)	7 (3%)	0
Thrombocytopenia	30 (14%)	10 (5%)	0	0
Decreased appetite	29 (13%)	0	13 (6%)	0
Peripheral oedema	28 (13%)	0	7 (3%)	0
Hyperglycaemia	26 (12%)	11 (5%)	4 (2%)	1 (0·5%)
Dyspnoea	26 (12%)	4 (2%)	3 (1%)	0
Pulmonary events‡	25 (12%)	5 (2%)	0	0
Vomiting	23 (11%)	1 (0·5%)	11 (5%)	1 (0·5%)
Pruritus	23 (11%)	0	8 (4%)	0
Asthenia	22 (10%)	2 (1%)	14 (7%)	1 (0·5%)

*Includes stomatitis, aphthous stomatitis, mouth ulceration, and tongue ulceration. †Includes all infections. ‡Includes pneumonitis, interstitial lung disease, lung infiltration, and pulmonary fibrosis.

Table 2: **Drug-related adverse events in at least 10% of patients (safety set)**

Discussion

Our findings show that median progression-free survival was greater in the everolimus plus octreotide LAR group than the placebo plus octreotide LAR group. Treatment

of advanced neuroendocrine tumours remains a clinical challenge because of the lack of effective options and the absence of well controlled randomised clinical trial data to support evidence-based practice. With few exceptions, chemotherapeutic drugs are not active in advanced non-pancreatic neuroendocrine tumours and are associated with substantial toxic effects. Thus, there is a need for new treatment options (panel).

Neuroendocrine tumours arise from various primary sites: primarily the small intestine, other sites of the gastrointestinal tract, and the lung.[19,20] The variable clinical course of advanced neuroendocrine tumours presents a major challenge for designing studies of appropriate power and duration.[21] Patients with neuroendocrine tumours often develop many metastases. Variability in the assessment of these metastases and potential differences in target lesion selection can result in discrepancies between local and central reviews,[22] presenting a challenge in assessing tumour response or progression during clinical trials. Discrepancies in radiological assessment between local and central reviews have resulted in loss of events and informative censoring in our trial. Informative censoring violates assumptions underlying the standard time-to-event analysis method and might obscure the progression-free survival treatment-effect estimate by central review.[23,24] The findings of our prespecified IPCW analysis done to assess this issue suggested that there was informative censoring, confounding the statistical interpretation of our primary endpoint analysis.

We previously showed that everolimus, with or without octreotide LAR, can be safely given to patients with advanced pancreatic neuroendocrine tumours.[12–14] Our present findings show that everolimus plus octreotide LAR compared with placebo plus octreotide LAR was associated with a clinically meaningful 5·1 month increase in median progression-free survival in patients with progressive advanced neuroendocrine tumours associated with a history of secretory symptoms. Consistent with these findings, treatment with everolimus plus octreotide LAR was associated with tumour shrinkage and stabilisation and significant reduction in biochemical markers of neuroendocrine tumours.

We did not collect outcomes reported by patients because we did not require them to have refractory symptoms at the time of study entry, as evidenced by the high number of patients who had a WHO performance status of 0 at the time of study entry, and because patients were allowed to receive octreotide LAR during the study for symptom control. Our study was not designed to assess the effect of everolimus on carcinoid-related symptoms.

Our study was affected by several factors, including inherent radiological challenges associated with the assessment of advanced neuroendocrine tumours, biological and clinical diversity of the population of patients, imbalances in baseline factors, and crossover design. Imbalances between study groups were noted in important prognostic baseline covariates, including primary tumour site, WHO performance status, and previous use of chemotherapy, all of which favoured the placebo plus octreotide LAR group and probably affected the primary outcome results. Despite this imbalance, everolimus was associated with a benefit on progression-free survival overall and across patient subgroups.

Our findings showing the efficacy of everolimus plus octreotide LAR in advanced neuroendocrine tumours are important because of the lack of effective anticancer treatment options. Efficacy of everolimus in this population will need confirmation in a future study. Together with clear evidence of benefit from the recently completed RADIANT-3[14] trial of everolimus in patients with advanced pancreatic neuroendocrine tumours, our data support the efficacy of everolimus in a broad spectrum of advanced neuroendocrine tumours.

Panel: Research in context

Systematic review

We searched Medline for reports on clinical trials in advanced neuroendocrine tumours, with "mTOR" and "NET" as our primary search terms. We did not limit our search by date. We identified no previous randomised studies of mTOR inhibitors in the present population.

Interpretation

Evidence-based treatment of neuroendocrine tumours is a challenge to clinicians because of the lack of reliable data from large clinical trials. No approved antitumour drugs are available for treating progressive disease in patients with gastrointestinal or lung neuroendocrine tumours, consequently affecting the survival of patients. Therefore, our findings that show the efficacy of the mTOR inhibitor everolimus plus octreotide LAR in advanced neuroendocrine tumours are important. These data support the efficacy of everolimus for the treatment of patients with a broad spectrum of advanced neuroendocrine tumours.

Contributors

MEP, JDH, and EB recruited patients, interpreted the data, wrote the report, reviewed the drafts, and approved the final draft. MP interpreted the data, wrote the report, reviewed the drafts, and approved the final draft. DH provided substantial clinical data, interpreted the data, wrote the report, reviewed the drafts, and approved the final draft. REW served as medical monitor, collected the data, analysed the data, interpreted the data, wrote the report, reviewed the drafts, and approved the final draft. JK and VJ analysed the data, interpreted the data, wrote the report, reviewed the drafts, and approved the final draft. DL designed the study, analysed the data, interpreted the data, wrote the report, reviewed the drafts, and approved the final draft. EMW designed the study, recruited patients, collected the data, analysed the data, interpreted the data, wrote the report, reviewed the drafts, and approved the final draft. KO designed the study, analysed the data, interpreted the data, wrote the report, reviewed the drafts, and approved the final draft. EVC recruited patients, interpreted the data, wrote the report, reviewed the drafts, and approved the final draft. JCY conceived and designed the study, recruited patients, collected the data, analysed the data, interpreted the data, wrote the report, reviewed the drafts, and approved the final draft.

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Conflicts of interest

MEP has served as a consultant and has received honoraria and research funding from Novartis. EB has received honoraria or research funding from Novartis. MP has received research funding and is on the speaker's bureau for Novartis. DH is a consultant to and has received honoraria and research funding from Novartis. REW, JK, DL, and VJ are employees of and own shares in Novartis. EMW is a consultant to Novartis. KO serves on advisory boards of and receives honoraria from Novartis, Pfizer, and Ipsen. EVC has received research funding from Novartis. JCY is a consultant to Novartis and has received research funding from Novartis. JDH declares no conflicts of interest.

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O^6-Methylguanine DNA Methyltransferase Deficiency and Response to Temozolomide-Based Therapy in Patients with Neuroendocrine Tumors

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Abstract

Purpose: Recent studies suggest that temozolomide has activity in neuroendocrine tumors. Low levels of the DNA repair enzyme, O^6-methylguanine DNA methyltransferase (MGMT), are associated with sensitivity to temozolomide in other tumor types. We evaluated the prevalence of MGMT deficiency in neuroendocrine tumors and correlated MGMT deficiency with treatment response to temozolomide-based regimens.

Experimental Design: The prevalence of MGMT deficiency, measured by immunohistochemistry, was assessed in 97 archival neuroendocrine tumor specimens. Rates of treatment response and survival were next evaluated in a cohort of 101 consecutive neuroendocrine tumor patients who had received treatment with a temozolomide-based regimen at one of three institutions. MGMT expression was directly correlated with treatment response in 21 patients who had available tumor tissue and response data.

Results: In archival specimens, MGMT deficiency was observed in 19 of 37 (51%) pancreatic neuroendocrine tumors and 0 of 60 (0%) carcinoid tumors ($P < 0.0001$). In the clinical cohort, 18 of 53 (34%) patients with pancreatic neuroendocrine tumors but only 1 of 44 (2%) patients with carcinoid tumors ($P < 0.001$) experienced a partial or complete response to temozolomide-based therapy. Among 21 patients with evaluable tumor tissue who had also received treatment with temozolomide, 4 of 5 patients with MGMT-deficient tumors (all pancreatic neuroendocrine tumors) and 0 of 16 patients with tumors showing intact MGMT expression responded to treatment ($P = 0.001$).

Conclusions: MGMT deficiency, measured by immunohistochemistry, is more common in pancreatic neuroendocrine tumors than in carcinoid tumors as is treatment response to temozolomide-based therapy. Absence of MGMT may explain the sensitivity of some pancreatic neuroendocrine tumors to treatment.

The alkylating agents streptozocin or dacarbazine are commonly incorporated in chemotherapy regimens for patients with advanced neuroendocrine tumors (1–7). Temozolomide is an alkylating agent initially developed as an oral and more easily tolerated alternative to dacarbazine. Initial clinical studies done with temozolomide showed clear evidence of activity in both melanoma and glioma (8–10). Recently, temozolomide has also been shown to have moderate activity in patients with advanced neuroendocrine tumors.

In an initial prospective study, treatment with temozolomide and thalidomide was associated with objective responses in 5 of 11 (45%) patients with pancreatic neuroendocrine tumors and 1 of 14 patients with carcinoid tumors (11). In a second prospective study, treatment with temozolomide and bevacizumab was associated with tumor responses in 4 of 17 (24%) patients with pancreatic neuroendocrine tumors and 0 of 12 patients with carcinoid tumors (12). Both regimens incorporated a dose-intense temozolomide regimen of 150 mg/m^2/d for 7 days administered on an every other week schedule.

Retrospective series further support the use of temozolomide in neuroendocrine tumors. In a series of 36 patients treated with temozolomide monotherapy, tumor regression was observed in 31% of bronchial carcinoid tumors and 8% of pancreatic neuroendocrine tumors (13). In small, retrospective series of patients with pancreatic neuroendocrine tumors, combination therapy with temozolomide and capecitabine

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Translational Relevance

Expression of the DNA repair enzyme MGMT correlates with treatment response to temozolomide in several tumor types. Recent evidence suggests that temozolomide-based therapy has activity in neuroendocrine tumors. In our study, we first observed that MGMT deficiency is more common in pancreatic neuroendocrine tumors than in carcinoid tumors. We next investigated the efficacy of temozolomide-based therapy in a large cohort of 101 neuroendocrine tumor patients. We found that the rate of treatment response to temozolomide-based therapy is significantly higher in pancreatic neuroendocrine tumors than in carcinoid tumors, a finding that is consistent with a higher prevalence of MGMT deficiency in this tumor subtype. In patients with available tumor tissue who had received treatment with temozolomide, MGMT expression directly correlated with treatment response. Neuroendocrine tumors have historically been considered resistant to standard cytotoxic chemotherapy regimens. Our findings confirm the activity of temozolomide-based regimens in pancreatic neuroendocrine tumors and suggest that approximately one-third of pancreatic neuroendocrine tumor patients will respond to such treatment. Our study further suggests that the tumors of the subgroup of patients who respond are characterized by MGMT deficiency as measured by immunohistochemical techniques. Prospective trials to validate immunohistochemical MGMT status as a predictive marker of response to temozolomide-based therapy in pancreatic neuroendocrine tumors are warranted.

has been associated with a tumor response rates of 59% to 71% (14, 15).

The cytotoxic effect of temozolomide has been attributed to its ability to induce DNA methylation at the O^6 position of guanine. Methylation of guanine results in DNA mismatch, ultimately resulting in apoptosis and tumor cell death (16). The sensitivity of tumor cells to alkylating agents, including temozolomide, has been associated with decreased levels of the DNA repair enzyme, O^6-methylguanine DNA methyltransferase (MGMT), which, through its ability to restore DNA to its normal form, can prevent chemotherapy-induced cell death (17). Among patients with either advanced melanoma or glioblastoma treated with temozolomide, loss of tumoral MGMT expression was associated with an improvement in survival (18–22).

We postulated that differences in MGMT expression might explain the sensitivity of some neuroendocrine tumors to temozolomide-based therapy. Previous studies evaluating the prognostic or predictive value of immunohistochemical MGMT expression have used various criteria to categorize tumors as having absent, low, or intact of MGMT (19, 23–26). To minimize potential subjectivity in our analysis, we used a prospective classification scheme describing tumors as either MGMT deficient (no detectable expression of MGMT in tumor cells) or MGMT intact. We first evaluated the prevalence of MGMT deficiency in a cohort of 97 archival tissue specimens comprising carcinoid and pancreatic neuroendocrine tumors. We next evaluated whether patterns of treatment response in 101 neuroendocrine tumor patients treated with temozolomide-based regimens at our institutions matched the observed patterns of MGMT deficiency in these tumor subtypes. Finally, we correlated MGMT expression with treatment response in a subset of 21 of these patients with available neuroendocrine tumor tissue specimens.

Materials and Methods

Evaluation of MGMT status in archival tissue specimens. Archival neuroendocrine tumor tissue specimens were identified through a review of pathology records at Brigham and Women's Hospital. Additional tumor blocks were requested for consenting patients who had received temozolomide-based therapy using an institutional review board-approved protocol. Paraffin sections (4 μm) were used for immunohistochemical staining. Tissue sections were incubated for 60 min at 60°C, deparaffinized, and rehydrated in graded ethanol solutions. Endogenous peroxidase activity was blocked by incubating the slides in 3% H_2O_2 for 10 min. The slides were then rinsed under running water for 5 min. Heat-induced epitope retrieval was done using a microwave oven at 199°F for 30 min in preheated 10 mmol/L citrate buffer (pH 6.0). The slides were then transferred to PBS. The tissue sections were then blocked with 1.5% horse serum for 15 min and incubated for 1 h at room temperature in a humid chamber with mouse monoclonal antibody to MGMT (1:25 dilution; clone MT 3.1; Lab Vision), a biotinylated secondary antibody (mouse IgG), and then avidin-horseradish peroxidase (Vectastain Elite ABC Kit; Vector Laboratories) according to the manufacturer's instructions. The slides were washed in PBS between incubations. Tissue sections were developed using 3,3′-diaminobenzidine (Sigma) as a substrate and counterstained with Gill's hematoxylin (Fisher Scientific) according to the manufacturers' instructions.

Immunohistochemical MGMT expression was measured in a blinded fashion by two pathologists (M.S.R. and J.L.H.) who reviewed all cases concurrently at a multiheaded microscope. Nuclear MGMT expression was scored as either "intact" or "deficient" in tumor cells using a prospective classification scheme. Tumors were scored as "intact" when there was nuclear staining for MGMT in any tumor cells. Tumors were scored as "deficient" when there was a complete absence of nuclear staining for MGMT in all tumor cells. Nonneoplastic cells (lymphocytes, stromal cells, and endothelial cells) served as an internal positive control in all tissue sections. The MGMT expression status was then correlated with tumor type and treatment outcome.

Table 1. Immunohistochemical MGMT expression in neuroendocrine tumors

Tumor type	*n*	MGMT deficient, *n* (%)	MGMT intact, *n* (%)
Pancreatic neuroendocrine	37	19 (51)	17 (49)
Nonfunctional	24	13	11
Insulinoma	10	3	7
Gastrinoma	2	2	0
Glucagonoma	1	1	0
Carcinoid	60	0	60 (100)*
Lung	40	0	40
Typical	20	0	20
Atypical	20	0	20
Small intestine	20	0	20

*$P < 0.0001$.

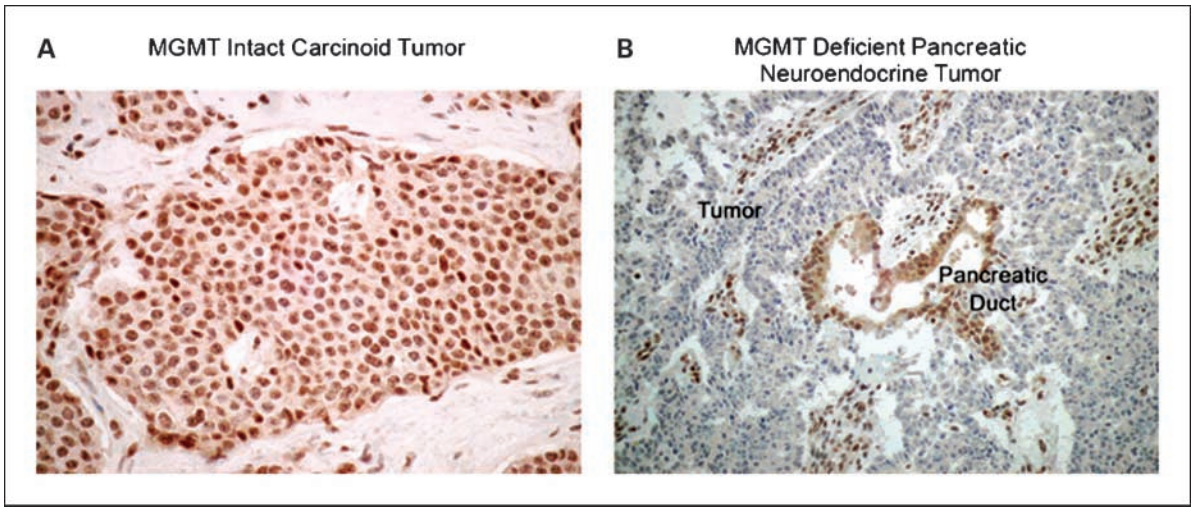

Fig. 1. Representative MGMT staining in carcinoid and pancreatic neuroendocrine tumors.

Identification of neuroendocrine patients who had received temozolomide-based therapy. We examined patients with locally advanced or metastatic neuroendocrine tumors who received temozolomide-based therapy either as part of prospectively conducted clinical trials or off-protocol at the discretion of the treating physician. Patients were treated at one of three institutions: Dana-Farber Cancer Institute, Massachusetts General Hospital, or Beth Israel Deaconess Medical Center. Patients were identified either through review of two clinical trials that included temozolomide or through an institutional review board-approved protocol in which patients provide informed consent for the use of medical records, biospecimens, and clinical outcome data for medical research purposes. Medical records and clinical trial records were used to obtain demographic and treatment information as well as to assess response to temozolomide-based therapy.

Assessment of response and survival. For all patients in this analysis, radiologic response was measured using Response Evaluation Criteria in Solid Tumors. Patients who had enrolled on prospective, phase II studies underwent baseline staging computed tomography scans within 4 weeks of treatment initiation, and every 8 weeks thereafter. Response measurements for patients who received temozolomide-based therapy outside of a study setting were obtained using the nearest pretreatment computed tomography scan and subsequent scans obtained as part of routine clinical care. Biochemical response was measured based on baseline chromogranin A levels obtained before initiation of temozolomide-based therapy. Patients were considered to have a partial biochemical response if there was a $\geq$50% reduction in plasma chromogranin A from the baseline level on two successive measurements. Overall survival (OS) was defined as the time from initiation of temozolomide-based treatment until death from any cause. Progression-free survival (PFS) was defined as the time from initiation of temozolomide therapy to the date of documented progression or death from any cause. OS and PFS were calculated using the Kaplan-Meier method.

Results

We first evaluated MGMT expression in a cohort of 97 archival neuroendocrine tumor specimens and compared the prevalence of MGMT deficiency in pancreatic neuroendocrine and carcinoid samples (Table 1; Fig. 1). Among 37 pancreatic neuroendocrine tumors, 19 (51%) were MGMT deficient. Absence of MGMT was observed in 13 of 24 nonfunctional pancreatic neuroendocrine tumors, 3 of 10 insulinomas, 2 of 2 gastrinomas, and 1 of 1 glucagonoma. In contrast, MGMT staining was intact in all 60 carcinoid tumors, comprising 20 typical bronchial carcinoid tumors, 20 atypical bronchial carcinoid tumors, and 20 small intestine carcinoid tumors ($P < 0.0001$). Heterogeneous staining for MGMT was observed in our study; tumors with heterogeneous staining were classified as MGMT "intact" according to the classification scheme. We noted particularly prominent heterogeneity in three atypical bronchial carcinoid tumors, suggesting that a significant subpopulation of cells in these tumors was MGMT deficient.

To evaluate whether patterns of treatment response might mirror the prevalence of MGMT deficiency in these tumor types, we next identified 101 patients who had received temozolomide-based therapy for neuroendocrine tumors and recorded treatment outcome according to tumor type (Table 2). The patient cohort had a median age of 57 years and had been diagnosed a median of 19.5 months before initiating treatment with temozolomide. Fifty-three patients had pancreatic neuroendocrine tumors, 44 had carcinoid tumors, and 4 had pheochromocytoma/paraganglioma. The majority of patients had received one or more systemic treatments for their malignancy before receiving treatment with temozolomide.

Of the 101 patients who received temozolomide-based therapy, 63 were treated as part of one of two prospective, single-arm, phase II clinical trials. These trials examined either the combination of temozolomide and thalidomide or temozolomide and bevacizumab. Within the clinical trials, temozolomide was administered at a dose of 150 mg/m^2/d in both regimens; thalidomide was administered at doses ranging from 200 to 400 mg/d, and bevacizumab at a dose of 5 mg/kg intravenously every other week. Similar regimens and starting doses were used in the majority of patients receiving temozolomide-based treatment outside of the formal study setting.

No significant differences in tumor response rates were observed based on the type of temozolomide regimen administered. Moreover, patients who received temozolomide as part of a clinical trial appeared to experience a similar objective response rate when compared with those who were treated outside of a clinical trial. A marked difference in

response rates was observed, however, between pancreatic neuroendocrine tumors and carcinoid tumors. Among 53 patients with pancreatic neuroendocrine tumors, 18 (34%) experienced partial responses to therapy as defined by Response Evaluation Criteria in Solid Tumors. In contrast, only 1 of 44 (2%) patients with carcinoid tumors experienced an objective response ($P < 0.001$); the single responder had metastatic well-differentiated bronchial carcinoid tumor. One of 4 patients with pheochromocytoma/paraganglioma responded to treatment.

The median PFS was 13.6 months for pancreatic neuroendocrine tumor patients and 9.6 months for patients with carcinoid tumors who received temozolomide ($P = 0.12$; Fig. 2A). Median OS was 35.3 months for patients with pancreatic neuroendocrine tumors and 19.4 months for patients with carcinoid tumors ($P = 0.07$; Fig. 2B).

In light of the parallel patterns of MGMT deficiency and treatment response among carcinoid and pancreatic neuroendocrine tumors, we postulated that MGMT expression might directly correlate with response to temozolomide therapy. We therefore examined the effect of immunohistochemical MGMT expression on clinical outcomes among 21 temozolomide-treated patients, comprising all patients for whom both clinical data and archival, paraffin-embedded specimens were available. Tumors from 16 of the treated patients (13 carcinoid tumors and 3 pancreatic neuroendocrine tumors) showed intact MGMT expression. None of these 16 patients experienced radiologic or biochemical responses to temozolomide. Five patients had tumors that were MGMT deficient; all five tumors were pancreatic neuroendocrine tumors. Four of these 5 (80%) patients experienced partial radiologic responses to treatment ($P = 0.001$); 4 of 5 also experienced biochemical (chromogranin A) responses. One patient who did not experience a radiologic response experienced a chromogranin A response; conversely, one of the radiologic responders did not have a chromogranin A response.

Among those patients who received temozolomide-based therapy, the median PFS for patients whose tumors showed intact MGMT expression was 9.3 months compared with 19.2 months for patients with MGMT-deficient tumors (Fig. 3A; $P = 0.11$). The median OS for patients whose tumors showed intact MGMT expression was 19.1 months; the median OS for patients with MGMT deficient tumors has not been reached (Fig. 3B).

Table 2.

(A) Patient characteristics and treatment response

Characteristics	*n*	Radiologic response, *n* (%)	Biochemical response (baseline elevated), *n* (%)
Tumor type			
Pancreatic neuroendocrine	53	18/53 (34)	16/32 (50)
Carcinoid tumors	44	1/44 (2)	6/27 (22)
Lung	8	1/8 (13)	3/8 (11)
Small bowel	19	0	1/19 (4)
Other/unknown	17	0	0
Paraganglioma/pheochromocytoma	4	1/4 (25)	2/2 (100)
Gender			
Male	51	10/51 (20)	9/31 (30)
Female	50	10/50 (20)	15/30 (50)
Median age	57		
Treatment regimen			
Temozolomide/thalidomide	44	8/44 (18)	14/25 (56)
Temozolomide/bevacizumab	52	11/52 (21)	9/33 (27)
Temozolomide/xeloda	1	1/1 (100)	1/1 (100)
Temozolomide alone	4	0/4 (0)	0/4 (0)
Treatment status			
Phase II study	63	12/63 (19)	11/37 (30)
Off-study	38	8/38 (21)	13/24 (54)
Median time from diagnosis (mo)	19.5		
No. prior systemic antitumoral treatments*			
0	44	12/44 (27)	11/30 (37)
1	35	3/35 (8)	10/19 (53)
2	6	2/6 (33)	2/5 (40)
3	6	2/6 (33)	1/5 (20)
4	1	1/1 (100)	NA

(B) MGMT status and treatment response

MGMT status	*n*	Radiologic response, *n* (%)	Biochemical response (baseline elevated), *n* (%)
MGMT intact †	16	0/16 (0)	0/10 (0)
MGMT deficient ‡	5	4/5 (80)	4/5 (80)

*Prior treatment data not available for 9 patients.
†Thirteen of 16 tumors with intact MGMT expression were carcinoids; 3 of 16 were pancreatic neuroendocrine tumors.
‡All 5 MGMT-deficient tumors were pancreatic neuroendocrine tumors.

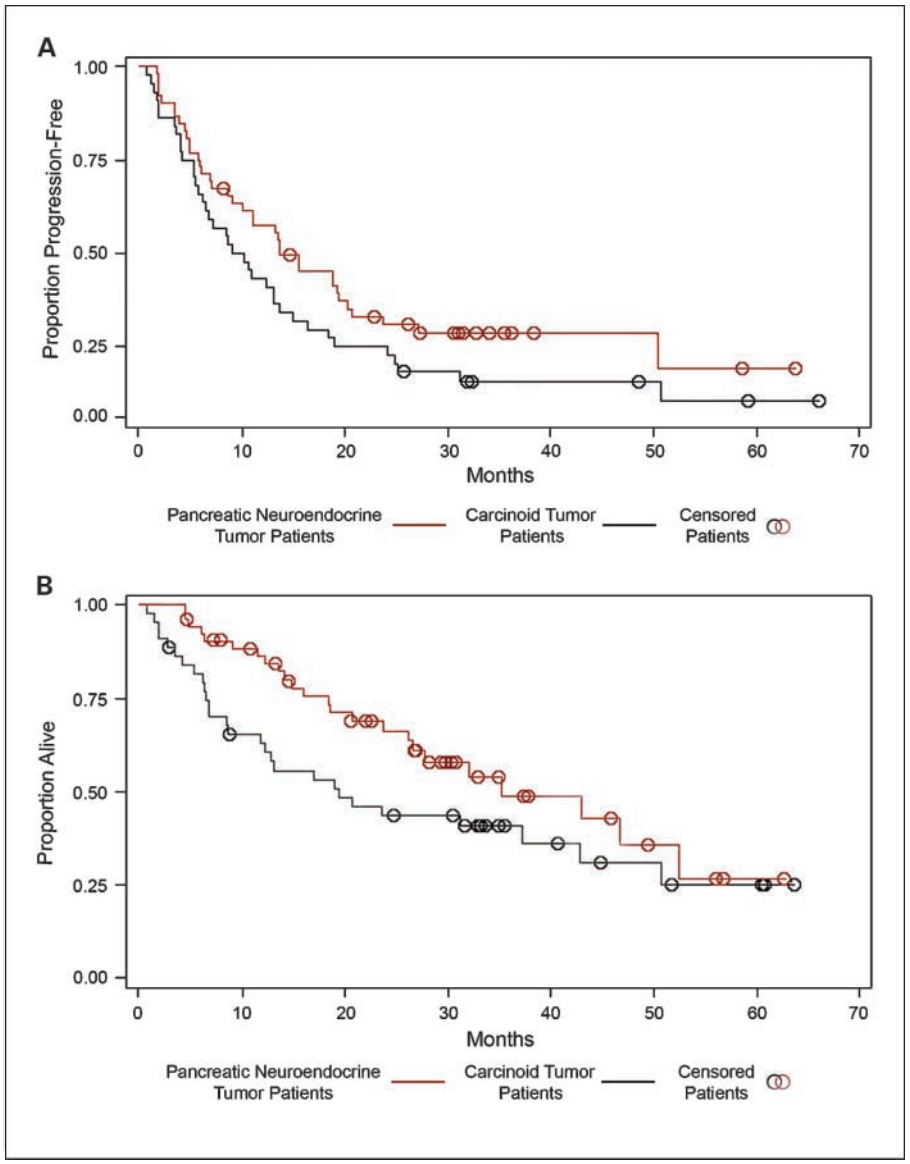

Fig. 2. PFS and OS for carcinoid and pancreatic neuroendocrine tumor patients treated with temozolomide-based therapy. *A,* median PFS was 13.6 mo for pancreatic neuroendocrine tumor patients and 9.6 mo for carcinoid tumor patients ($P = 0.12$). *B,* median OS was 35.3 mo for pancreatic neuroendocrine tumor patients and 19.4 mo for carcinoid tumor patients ($P = 0.07$).

Discussion

In a large cohort of archival neuroendocrine tumor specimens, we found that MGMT deficiency, as measured by immunohistochemistry, was more common in pancreatic neuroendocrine tumors than in carcinoid tumors. Consistent with this difference, we found that 34% of patients with pancreatic neuroendocrine tumors treated with temozolomide-based regimens experienced a Response Evaluation Criteria in Solid Tumors-defined radiologic tumor regression, whereas responses in carcinoid tumor patients were rare. MGMT deficiency was directly associated with treatment response to temozolomide in the subgroup of 21 treated patients who also had available tumor tissue.

Like temozolomide, streptozocin and dacarbazine induce methylation at the O^6 position of guanine (27–30). This common cytotoxic mechanism suggests that the mechanisms of drug resistance for these agents may also be similar and that the ability of MGMT to repair treatment-induced formation of O^6 methylguanine may contribute to drug resistance to all three drugs. Our observations that temozolomide-based therapy is more effective in pancreatic neuroendocrine tumors than in carcinoid tumors in fact mirror earlier results with the alkylating agents streptozocin and dacarbazine.

In an initial randomized trial, the combination of streptozocin and doxorubicin was associated with a combined biochemical and radiologic response rate of 69% in patients with pancreatic neuroendocrine tumors (5). In a retrospective analysis of 84 pancreatic neuroendocrine tumor patients treated with streptozocin, 5-fluorouracil, and doxorubicin, using more formal radiologic response criteria, the overall response rate was 39% (4). Dacarbazine was associated with an overall response rate of 33% in patients with pancreatic neuroendocrine tumors in a phase II study (6). The response rate of 34% observed with temozolomide in the current study is similar to that observed in these prior studies.

Response rates associated with these alkylating agents in carcinoid tumors are lower. In a recent trial, 249 patients with advanced carcinoid tumors were randomized to receive either streptozocin/5-fluorouracil or 5-fluorouracil

/doxorubicin (7). The response rates associated with these regimens were 16% and 15.9%, respectively. The reported response rates associated with single-agent dacarbazine in carcinoid tumors are 8% to 16% (2, 7). Only a single carcinoid tumor patient (2%) responded to temozolomide-based therapy in our series.

Our results are similar to those of a smaller study of temozolomide monotherapy in 36 patients with neuroendocrine tumors (13). As in our study, 4 of 5 responding patients in the monotherapy study had low MGMT expression; responses were uncommon in patients with high MGMT expression. In contrast to our observations, however, temozolomide monotherapy was associated with an overall response rate of 31% (4 of 13) in patients with bronchial carcinoid tumors. We identified only 8 bronchial carcinoid tumor patients in our series, limiting our ability to more formally evaluate the efficacy of temozolomide in this subpopulation. Interestingly, the single carcinoid patient who responded to temozolomide in our study had a bronchial carcinoid tumor. Although several tumors classified as MGMT "intact" showed heterogeneous staining in our study, we observed a markedly heterogeneous pattern of MGMT expression in three atypical bronchial carcinoid tumors, providing a possible explanation for the sensitivity of some carcinoid tumors to temozolomide.

Streptozocin-based therapy has been associated with improved OS in patients with pancreatic neuroendocrine tumors (5). We observed trends toward improved PFS and OS among temozolomide-treated patients with pancreatic neuroendocrine tumors when compared with treated patients with carcinoid tumors in our study. Survival comparisons in our cohort are limited by both the retrospective nature of our analysis and potential differences in the treated subpopulations. Nevertheless, given the often similar natural history of patients with these malignancies, our observations raise the possibility that the higher observed rate of treatment response may also translate into improved survival in patients with pancreatic neuroendocrine tumors. Although we also observed a trend toward improved PFS and OS in patients with MGMT-deficient compared with MGMT-intact tumors, we cannot rule out the possibility that MGMT status had an independent effect on survival. Prospective, randomized studies will be necessary to confirm these associations.

There remains considerable controversy regarding the optimal method of MGMT analysis in clinical studies. Direct

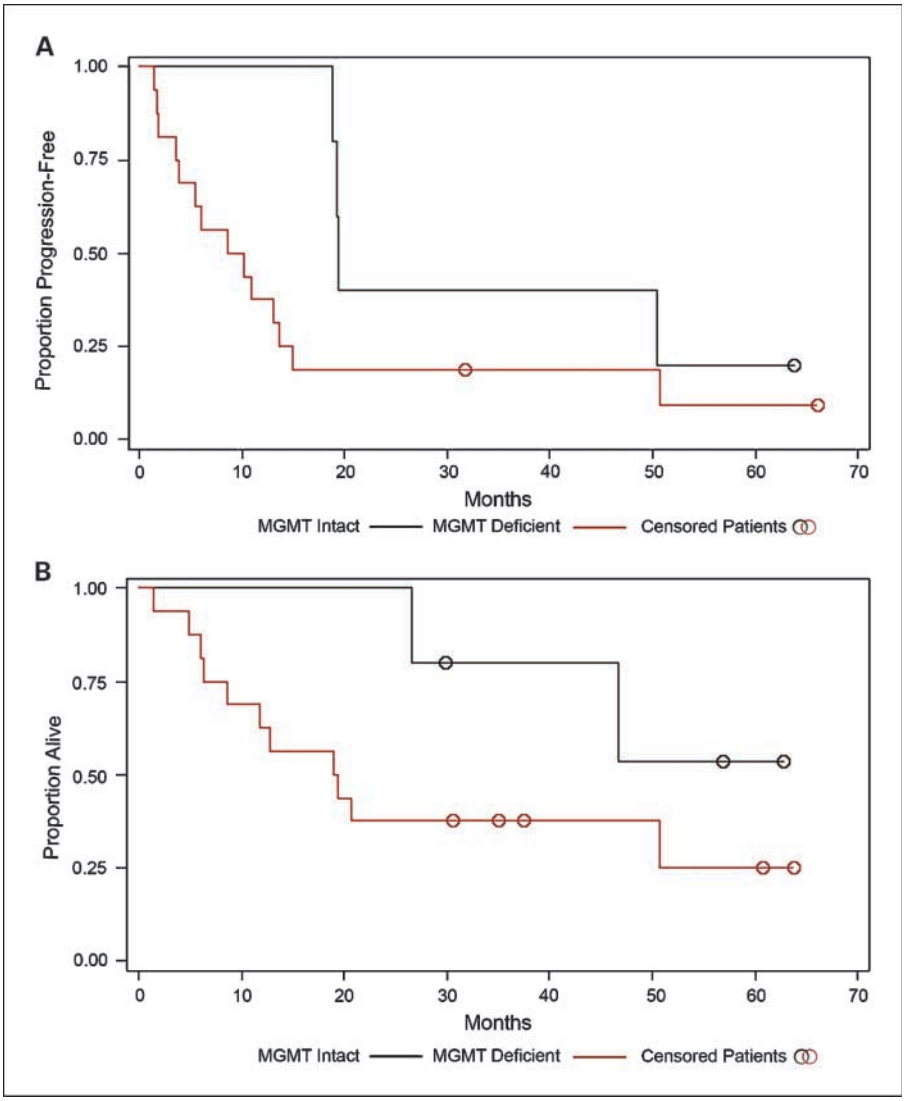

Fig. 3. PFS and OS in patients with MGMT-intact or MGMT-deficient neuroendocrine tumors treated with temozolomide-based therapy. *A,* median PFS was 19.2 mo for MGMT-deficient neuroendocrine tumors and 9.3 mo for MGMT-intact tumors ($P = 0.11$). *B,* median OS for patients with MGMT-deficient tumors was not reached; median OS for patients with MGMT-intact tumors was 19.1 mo ($P = 0.14$).

analysis of MGMT enzymatic activity generally requires use of carefully preserved frozen tissue or cell lysates and is not readily applicable to analysis of archival tumor samples from large clinical studies (31–33). Epigenetic silencing of the *MGMT* gene by CpG island promoter methylation is a common mechanism of *MGMT* gene regulation, and promoter methylation status, assessed by methylation-specific PCR, has been widely used as a surrogate marker of MGMT activity in clinical specimens (34). In patients with glioblastoma, *MGMT* promoter methylation has been associated with improved survival and benefit from temozolomide in most, although not all, studies (20, 21, 23, 35–37). Direct measurement of MGMT protein expression using immunohistochemistry, as was done in our study, is the technically easiest and perhaps the most commonly used technique to measure MGMT status in tumor samples. As with *MGMT* promoter methylation, low levels of immunohistochemical MGMT expression have been associated with improved response to temozolomide in glioblastoma in many studies, although correlations have not always been consistent (19, 22–25, 38).

Our observation that MGMT deficiency is more common in pancreatic neuroendocrine than in carcinoid tumors would suggest that MGMT promoter methylation status may also be more prevalent in pancreatic neuroendocrine tumors. However, previously reported studies of CpG island methylation in neuroendocrine tumors have found either no significant difference in MGMT promoter methylation rates between carcinoid and pancreatic neuroendocrine tumors or higher rates of promoter methylation in carcinoid tumors compared with pancreatic neuroendocrine tumors (39, 40). A poor correlation between *MGMT* promoter methylation and immunohistochemical expression of MGMT has been reported in several studies directly comparing these two methods (41–43). One study evaluating 31 glioblastoma samples found evidence of MGMT promoter methylation in 61% of samples but low level immunohistochemical MGMT expression (<20% nuclear staining) in only 31% (41). In a second study, substantial numbers of MGMT-positive cells were detected in the majority (73%) of tumor specimens carrying a methylated promoter (42). Tumor heterogeneity, as well as the presence of endothelial cells and other nonneoplastic components expressing MGMT in tumor samples, may have contributed to the discordant results observed in these studies.

We sought to minimize these limitations in our study by prospectively using a strict definition for MGMT deficiency, in which specimens were only considered deficient if they showed complete absence of detectable MGMT in tumor cells by immunohistochemistry. We further specifically identified nonneoplastic components of the tumors using these elements as positive internal controls. Nevertheless, technical limitations and interobserver variability remain a concern in the interpretation of MGMT immunohistochemical assays. We also cannot rule out the possibility that mechanisms other than MGMT expression affect neuroendocrine tumor sensitivity to temozolomide. Parallel DNA repair mechanisms, including the base excision repair system, may affect temozolomide sensitivity, resulting in an imperfect correlation between MGMT expression and treatment response (44, 45).

In summary, MGMT deficiency, as measured immunohistochemically, appears to be more common in pancreatic neuroendocrine tumors than in carcinoid tumors. Consistent with this finding, in a retrospective analysis, we observed a 34% response rate to temozolomide-based therapy in pancreatic neuroendocrine tumors compared with 2% in carcinoid tumors. MGMT deficiency was directly associated with temozolomide response in the patient subgroup with available tumor tissue and treatment data. Our findings suggest that MGMT status could be used as a predictive marker to identify neuroendocrine tumor patients who are likely to respond to treatment with alkylating agents. Standardization of techniques to assess MGMT status in tumor tissue, together with prospective trials to confirm a correlation between MGMT status and treatment response in neuroendocrine tumor patients treated with alkylating agents, is warranted.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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Clinical Cancer Research

O^6-Methylguanine DNA Methyltransferase Deficiency and Response to Temozolomide-Based Therapy in Patients with Neuroendocrine Tumors

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CDKN2A as transcriptomic marker for muscle-invasive bladder cancer risk stratification and therapy decision-making

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Deletions of the cell cycle control gene *CDKN2A* are described as progression markers of non-muscle invasive bladder cancer and to be associated with fibroblast growth factor 3 (*FGFR3*) mutations. The prognostic role of *CDKN2A* RNA expression in muscle invasive bladder cancer (MIBC) is under discussion. In 80 MIBC patients (m/f 60/20) who underwent radical cystectomy the expression of *CDKN2A* and *FGFR3* was examined with qRT-PCR (test cohort). The MDA cohort (n = 57) and the TCGA cohort (n = 365) served for validation. The expression of drug target genes and TCGA molecular subtypes was correlated with *CDKN2A* expression. In the test cohort *CDKN2A*^{high} patients (n = 8; 10.0%) had a significantly shorter recurrence-free (p = 0.018) and disease-specific (p = 0.006) survival compared to the rest of the cohort. A similar stratification was seen in the validation cohorts (*CDKN2A*^{high}: n = 7, 12.3%, p = 0.001; n = 46, 12.6%, p = 0.011). In the TCGA cohort these patients had a comparably low expression of drug target genes. The expression of *CDKN2A* significantly differed among TGCA molecular subtypes. 71.7% of *CDKN2A*^{high} were TCGA basal squamous tumours but also show divergent molecular features compared to this group. In summary *CDKN2A* RNA expression-based risk stratification of MIBC allows the identification of a *CDKN2A*^{high} poor prognosis group with low expression of drug target genes.

For several decades radical cystectomy (RC) is the standard therapy of muscle invasive bladder cancer (MIBC). Yet, due to a high recurrence rate, 5-year overall survival (OS) of patients with locally advanced tumours is only around 50%[1]. In the hospital routine decisions on adjuvant, neoadjuvant and palliative medication still mainly rely on clinical parameters. Though deemed crucial in terms of risk stratification and identification of patients in need for a more aggressive treatment, molecular profiling for individual therapy decision-making is still in it's infancy in MIBC[2,3]. Furthermore, expression data can give valuable information about drug target gene expression[4–6].

In the light of bladder cancer initiation several frequent genetic aberrations have been identified. Papillary/non muscle-invasive and non-papillary/muscle-invasive bladder cancer are typically seen as two different molecular entities[7]. In both groups alterations of "forerunner genes" are seen as an initial event. Whilst in papillary tumours, genetic alterations are mainly restricted to these genes, high risk NMIBC and MIBC often show alterations of major tumour suppressor genes as *RB1* or *TP53*[8,9].

Loss of heterozygosity (LOH) in the 9p region is one of this typical early events in the formation of bladder cancer and frequently occurs in non-invasive precursor lesions like hyperplasia, dysplasia or carcinoma *in situ*[10–13]. One of the genes found in this region is *CDKN2A*, which codes for the cell cycle control protein p16. LOH of

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parameter	total (n = 80)	*CDKN2A* low (n = 72)	*CDKN2A* high (n = 8)	p-value (Chi²)
Male Female	60 (75.0%) 20 (25.0%)	55 (76.4%) 17 (23.6%)	5 (62.5%) 3 (37.5%)	p = 0.389
Age	66 (46–93)	66 (46–85)	72 (54–93)	t-test p = 0.081
T2 T3 T4	19 (23.8%) 47 (58.8%) 14 (16.5%)	18 (25.0%) 42 (58.3%) 12 (16.7%)	1 (12.5%) 5 (62.5%) 2 (25.5%)	p = 0.677
N0 N1 Nx	48 (60.0%) 32 (40.0%) —	44 (61.1%) 28 (38.9%) —	4 (50.0%) 4 (50.0%) —	p = 0.543

Table 1. Patient characteristics of the test cohort.

parameter	total (n = 57)	*CDKN2A* low (n = 50)	*CDKN2A* high (n = 7)	p-value (Chi²)
Male Female	49 (85.9%) 8 (14.1%)	43 (86.0%) 7 (14.0%)	6 (85.7%) 1 (14.3%)	p = 0.984
Age	66 (41–89)	66 (41–89)	61 (41–85)	t-test p = 0.302
T1 T2 T3 T4	2 (3.5%) 12 (21.1%) 35 (61.4%) 8 (14.0%)	1 (2.0%) 12 (24.0%) 31 (62.0%) 4 (8.0%)	1 (14.2%) 0 (0.0%) 4 (57.1%) 2 (28.6%)	p = 0.086
N0 N1 N2	22 (38.6%) 9 (15.8%) 26 (45.6%)	19 (38.0%) 8 (16.0%) 23 (46.0%)	3 (42.9%) 1 (14.2%) 3 (42.9%)	p = 0.969

Table 2. Patient characteristics of the MDA cohort.

CDKN2A and decreased expression of the p16 protein are mainly described as a predictor of progression in non muscle-invasive bladder cancer (NMIBC)[14]. Homozygous deletion of *CDKN2A*, is also associated with muscle invasion in *FGFR3*-mutated (fibroblast growth factor receptor 3) tumours[15].

On the protein level a meta-analysis[16], including data from 17 immunohistochemistry studies with 1032 subjects, investigated the p16 expression in various disease stages and found a significant association between a low expression of p16 and recurrence-free survival in patients with all stages of bladder cancer. When stratifying for T stages this correlation was markedly stronger for NMIBC, but was not found for MIBC (≥T2). The same was found for progression-free survival (PFS). The authors concluded that the p16 expression is affected by clinicopathologic stage and its relevance is mainly to be seen in NMIBC.

Another study found altered p16 protein expression, defined as either no expression of p16 or a very strong p16 expression, to be associated with a worse outcome of MIBC[17]. These results support a more complex role of *CDKN2A* and the p16 protein in MIBC.

We therefore aimed to stratify patients with MBIC according to their *CDKN2A* expression. Since immunohistochemistry is limited in case of quantification and sample comparison, RNA-based methods like qRT-PCR or next generation sequencing are robust alternatives for quantification and stratification of gene expression. The value of *CDKN2A* mRNA expression has not been systematically investigated in MIBC, yet, but qRT-PCR has already proved to be a valuable tool to determine *CDKN2A* copy number status[18]. The *CDKN2A* RNA-expression-based risk stratification was validated in the MDA and the TCGA cohort. Furthermore we reanalysed TCGA data to reveal correlations of *CDKN2A* with drug target gene expression and molecular subtypes.

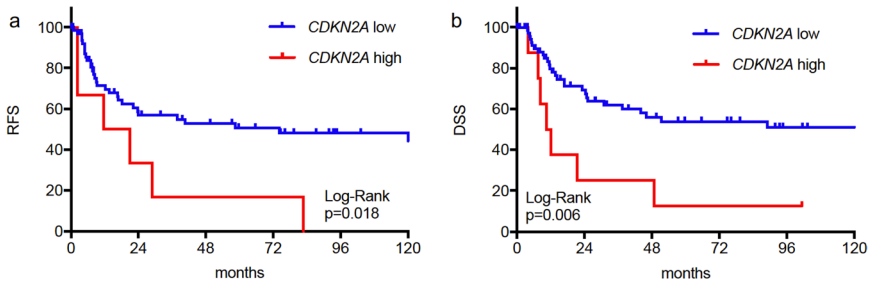
Results

***CDKN2A* RNA expression allows risk stratification of MIBC patients.** When stratifying for disease-specific death using the partition test, the test cohort of 80 patients with MIBC could be divided into two groups with different *CDKN2A* expression (*CDKN2A*^{high} with n = 8, 10.0%; *CDKN2A*^{low} with n = 72, 90.0%). Clinicopathologic data did not differ significantly between these groups (Table 1).

Kaplan-Meier analysis for recurrence-free survival (RFS, Fig. 1a) and disease-specific survival (DSS, Fig. 1b) showed significant differences (p = 0.018 and p = 0.006) between these groups, with *CDKN2A*^{high} having a worse prognosis (median RFS 16.3 months and median DSS 11.2 months) compared to patients with *CDKN2A*^{low} tumours (median RFS 74.2 months and median DSS 131.7 months).

By using the partition test in the MDA cohort of 57 bladder cancer patients a similar cut-off for *CDKN2A* expression could be defined. As in the test cohort, those patients with the highest *CDKN2A* expression (n = 7; 12.3%) had a worse prognosis (p = 0.001; median DSS 25.3 months, for *CDKN2A* median DSS was not reached; Fig. 2a).

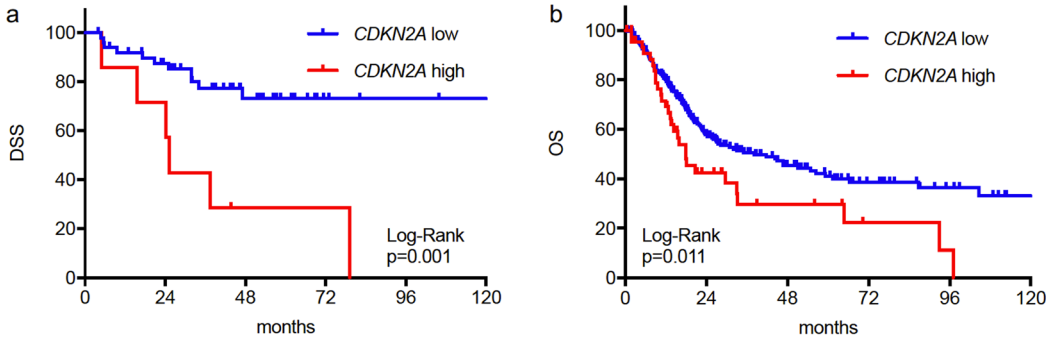
When applying the partition test to the TCGA cohort, again a small group of patients with the highest *CDKN2A* expression (n = 46; 12.6%) with a poor prognosis could be identified (p = 0.011; Fig. 2b). The median overall survival (OS) of *CDKN2A*^{high} was 18.0 months, compared to 38.2 months in the *CDKN2A*^{low} group



group	n	censored	event	median RFS
low	72	41	31	74.2
high	8	2	6	16.3

group	n	censored	event	median DSS
low	72	43	29	131.7
high	8	1	7	11.2

Figure 1. Stratification of the test cohort (n = 80) for *CDKN2A* expression identified a subgroup of 8/80 (10.0%) patients with the highest *CDKN2A* expression who had a much worse RFS (**a**) and DSS (**b**) compared to the rest of the cohort.



group	n	censored	event	median OS
low	50	39	11	n.d.
high	7	1	6	25.3

group	n	censored	event	median OS
low	319	186	133	38.2
high	46	17	29	18.0

Figure 2. Validation in (**a**) the MDA cohort and (**b**) the TCGA cohort confirmed the poor prognosis of a similar proportion of patients (**a**: 12,3% and **b**: 12,6%) with the highest *CDKN2A* expression. (n.d. = not defined).

(n = 319; 87.4%). Clinicopathologic data did not differ significantly between the *CDKN2A* expression groups in the MDA and the TCGA cohort (Table 2 and Table 3).

Further dissection of the bigger group of CDKN2A^{low} tumours in the test cohort and the TCGA cohort did not result in similar subgroup sizes, but yet resulted in different cut-offs with significant differences in prognosis, with patients with an intermediate expression of CDKN2A having a better prognosis as those with a low expression (Supplementary Figure 1).

Expression of drug target genes in dependence on *CDKN2A* expression. In the TCGA cohort there was a negative correlation between *CDKN2A* and *FGFR3* in all MIBC ($\rho = -0.406$; $p < 0.001$). In the test cohort there was also a trend towards a negative correlation between *FGFR3* and *CDKN2A* ($\rho = -0.217$, $p = 0.053$). Yet, inter-group comparison did not show a significant difference in *FGFR3* expression between *CDKN2A*^{low} and *CDKN2A*^{high} tumours ($p = 0493$; Fig. 3a). In the TCGA cohort the *FGFR3* expression differed significantly between the *CDKN2A* expression groups ($p < 0.001$; Fig. 3b).

Stratification for *CDKN2A* expression also showed a significantly lower expression of *ESR2* in *CDKN2A*^{high} tumours ($p < 0.001$, Fig. 3c). For the other tested drug target genes (*AR, ESR1, ERBB2, PDCD1, CD274* and *CTLA4*) no significant differences in the two *CDKN2A* expression groups were seen. Over all MIBC patients from the TCGA cohort *AR* was negatively correlated with *CDKN2A* expression ($\rho = -0.183$; $p = 0.004$) and *PDCD1*, *CD274* and *CTLA4* were positively correlated with *CDKN2A* expression ($\rho = 0.176$; $p < 0.001$; $\rho = 0.327$; $p < 0.001$; $\rho = 0.171$; $p < 0.001$). Detailed results are given in Supplementary Table 1.

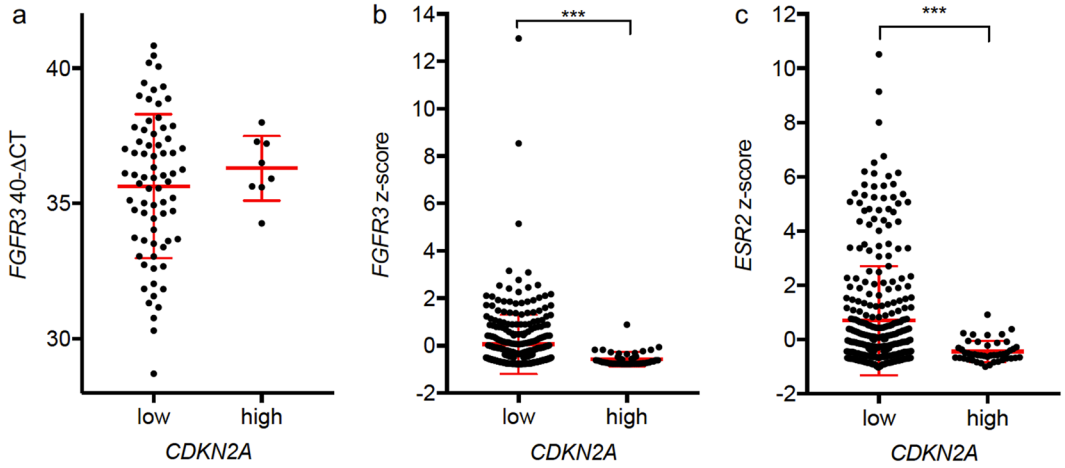


Figure 3. (**a**) In the test cohort there was no difference in the *FGFR3* expression in the *CDKN2A* expression groups. (**b**) In the TCGA cohort *FGFR3* was significantly lower expressed in $CDKN2A^{high}$ tumours. $CDKN2A^{low}$ tumours had also a higher expression of *ESR2* (**c**). (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

parameter	total (n = 365)	*CDKN2A* low (n = 319)	*CDKN2A* high (n = 46)	p-value (Chi^2)
Male Female	269 (73.7%) 96 (26.3%)	239 (74.9%) 80 (25.1%)	30 (65.2%) 16 (34.8%)	p = 0.162
Age	68 (34–90)	68 (34–90)	68 (44–90)	t-test p = 0.989
T2 T3 T4	118 (32.3%) 190 (52.1%) 57 (15.6%)	104 (32.6%) 164 (51.4%) 51 (16.0%)	14 (30.4%) 26 (56.6%) 6 (13.0%)	p = 0.785
N0 N1 Nx	217 (59.5%) 125 (34.2%) 23 (6.3%)	191 (59.9%) 108 (33.9%) 20 (6.2%)	26 (56.6%) 17 (36.9%) 3 (6.5%)	p = 0.908
Neoadjuvant treatment				p = 0.249
Yes	9 (2.47%)	9 (2.8%)	0 (0.0%)	
No	356 (97.5%)	310 (97.2%)	46 (100.0%)	
Adjuvant chemotherapy				p = 0.0582
Yes	62 (17.0%)	58 (18.2%)	4 (8.7%)	
No	158 (43.3%)	131 (41.1%)	27 (58.7%)	
NA	145 (39.7%)	130 (40.7%)	15 (32.6%)	
Adjuvant radiotherapy				p = 0.167
Yes	7 (1.9%)	7 (2.2%)	0 (0.0%)	
No	227 (62.2%)	193 (60.5%)	34 (73.9%)	
NA	131 (35.9%)	119 (37.3%)	12 (26.1%)	

Table 3. Patient characteristics of the TGCA cohort.

***CDKN2A* CNV status, downstream target expression and molecular subtypes.** In the TCGA cohort patients with $CDKN2A^{low}$ expression frequently had *CDKN2A* deletions (38.9%% homozygous deletion, heterozygous deletions: 26.0%) in comparison to more balanced genotypes in the $CDKN2A^{high}$ group (−1: 21.7%, balanced: 34.8%, +1: 43.5%, Supplementary Figure 2a). Chi^2 test proved this difference to by significant ($p < 0.001$). Vice versa, upon stratification of *CDKN2A* expression for *CDKN2A* CNV there was also a significant difference between CNV groups (Kruskal-Wallis $p < 0.001$, Supplementary Figure 2b).

Supplementary Figure 3a illustrates the *CDKN2A* expression in the *CDKN2A* expression groups of the TCGA cohort. *CDK4*, despite being negatively regulated by *CDKN2A*, showed a slightly, but not significantly higher expression in $CDKN2A^{high}$ ($p < 0.139$; Supplementary Figure 3b). *RB1*, which is regulated by *CDK4*, showed a significantly lower expression in $CDKN2A^{high}$ ($p < 0.001$, Supplementary Figure 3c) and the downstream transcription factor gene *E2F3* was significantly higher expressed in $CDKN2A^{high}$ tumours ($p < 0.001$, Supplementary Figure 3d). In the complete cohort a positive correlation was observed between *CDKN2A* and *CDK4* gene expression ($\rho = 0.222$, $p < 0.001$) and *E2F3* gene expression ($\rho = 0.212$, $p < 0.001$) and a negative correlation between *CDKN2A* and *RB1* $\rho = -0.479$ ($p < 0.001$).

When correlated with copy number status, there was a significant difference in the distribution of updated TCGA subtypes[19] (Chi^2 $p = 0.016$; Table 4 and Fig. 4a). Tumours with basal squamous and neuronal expression

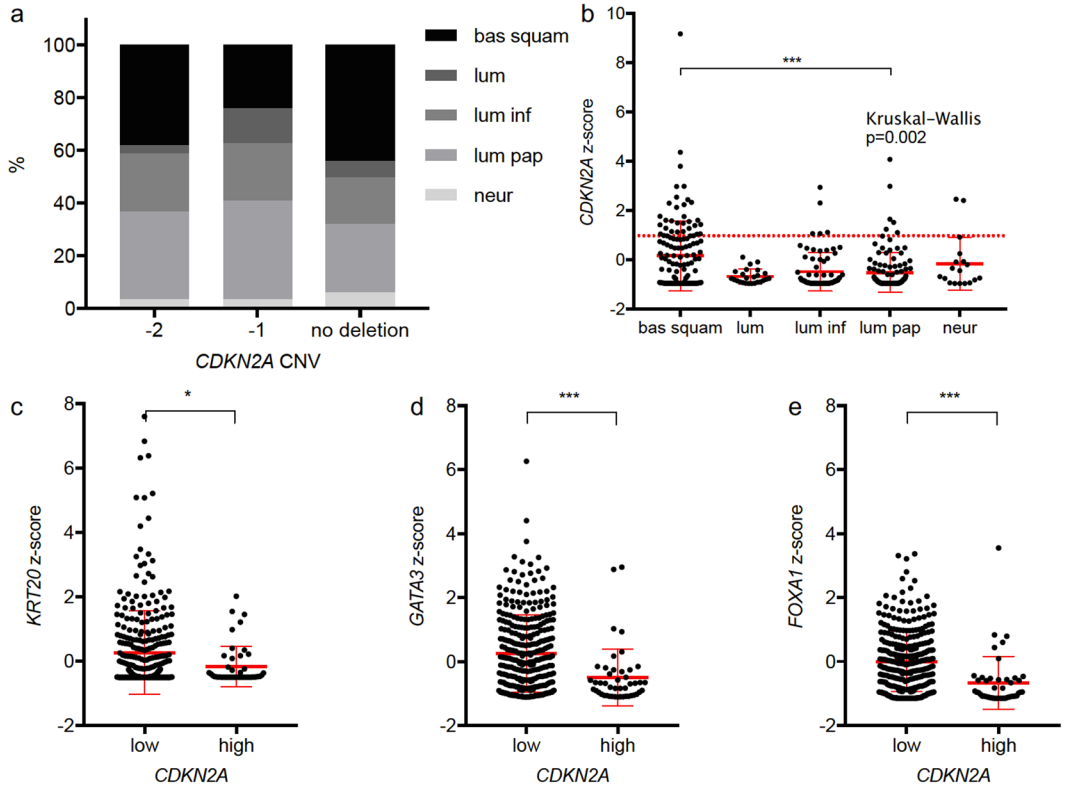


Figure 4. (**a**) In the TCGA cohort the distribution of TCGA RNA-expression subtypes significantly differs according to *CDKN2A* copy number status (Chi² 0.016). (**b**) Vice versa there were significant differences in the *CDKN2A* expression in these subtypes (bas squam = basal squamous, lum = luminal, lum inf = luminal infiltrated, lum pap = luminal papillary, neur = neuronal). Of the analyzed, typically subtype defining, genes, *KRT20*, *GATA3* and *FOXA1* showed a differing expression in the *CDKN2A* expression groups (**c**–**e**). (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

Copy number status	Subtypes					p-value (Chi²)
	Basal squamous	Luminal	Luminal infiltrated	Luminal papillary	Neuronal	
−2	47	4	27	41	4	p = 0.016
−1	22	12	20	34	3	
no deletion	65	9	26	38	9	

Table 4. Chi²-square test showed significant difference in the distribution of TCGA subtypes according to copy number status.

phenotype were overrepresented in the group of tumours with no deletion. Luminal tumours mainly had a homozygous deletion. Tumours from the basal squamous group on average showed the highest *CDKN2A* expression and tumours from the luminal group had a comparably low *CDKN2A* expression. Over all groups Kruskal-Wallis test showed a significantly different distribution, with 33 of 46 tumours (71.7%) in the $CDKN2A^{high}$ group being classified as basal squamous (Fig. 4b).

When looking at specific genes, typically determining RNA expression subtypes, *GATA3* ($\rho = -0.162$; $p = 0.002$) and *FOXA1* ($\rho = -0.299$; $p < 0.001$) showed an inverse correlation with the *CDKN2A* expression. Both genes also showed a significantly lower expression in $CDKN2A^{high}$ tumours compared to $CDKN2A^{low}$ tumours (both $p < 0.001$; Fig. 4d,e). Furthermore *KRT20* was significantly lower expressed in $CDKN2A^{high}$ tumours ($p = 0.028$; Fig. 4c). Detailed results are given in Supplementary Table 2.

Discussion

Deletions of *CDKN2A* and the underexpression of p16, the protein coded by *CDKN2A*, are well-investigated molecular risk factors for tumour progression in NMIBC. Based on this, one could conclude that deletion or underexpression of *CDKN2A*/p16 is also an indicator of increased aggressiveness and worse prognosis in MIBC. Yet, some data point to a more complex situation in MIBC. For instance, gene expression studies have shown distinct RNA expression patterns for Ta tumours and MIBC, with T1 tumours showing either one or the other signature[20]. To more deeply investigate the role of *CDKN2A* expression in tumour prognosis and its association with

drug target genes like *FGFR3*, we performed qRT-PCR expression profiling and reanalysis of existing *CDKN2A* and *FGFR3* RNA expression data of MIBC after RC. Compared to immunohistochemistry studies of p16, RNA-testing with qRT-PCR has the advantages of a higher dynamic width and a higher sensitivity. Furthermore qRT-PCR testing allows an observer-independent interpretation of quantifiably results.

In the three analyzed cohorts the groups with the highest expression of *CDKN2A* (10.0–12.6% of the examined patients) were identified to have a worse prognosis compared to the remaining patients. This is controversial to previous assumptions derived from findings in NMIBC, where deletion of *CDKN2A*, typically going along with a lowered expression, is deemed as a marker for poor prognosis[2,21,22].

However, already in 2004 it was shown that both a low and a high expression of the p16 protein can be a predictor of worse outcome after RC[17]. The overall prevalence of altered p16 protein expression was 54% of the analyzed tumours. The results of last-mentioned study show that both high and low expression of *CDKN2A* and p16, respectively, are associated with a worse outcome of MIBC patients. Though we could not find similar cut-off values in the different cohorts to distinguish between patients with low and intermediate expression of CDKN2A in the present study, patients with an intermediate expression seem to have the best prognosis, pointing to a diverse role of *CDKN2A* as prognosis marker in MIBC.

Functionally it is well known that impaired function or expression of p16, either due to *CDKN2A* deletion, mutation or hypermethylation, leads to cell cycle deregulation via overactivation of CDK4 and CDK6, which results in hyperphosphorylation of retinoblastoma protein (RB), the protein product of *RB1*. The subsequent liberation of E2F transcription factor family members mediates changes in gene expression, promoting the transition from G1 to S phase[23]. Besides this mechanism, loss of *RB1* also results in tumour formation and progression[24].

This close relation between *CDKN2A*/p16 and RB has been repeatedly described in urinary bladder cancer[25,26]. Yet, this does not intuitively explain why those MIBC with the highest *CDKN2A* expression show a poor prognosis. Sjödahl *et al.* reported two different genomic circuits operative in urothelial carcinomas: one defined by high *FGFR3* and *CCND1* expression, low *CDKN2A* expression, often associated with *CDKN2A* loss and the other one defined by *E2F3* amplifications and overexpression, *RB1* deletions and low expression and high *CDKN2A*/p16 expression[27]. Whilst the first circuit is mainly found in tumours termed urobasal A and urobasal B, the latter circuit was mainly associated with genomically unstable tumours[28,29]. These tumours also on the protein level typically showed no or low expression of KRT5 and KRT14, aberrant expression of KRT20 and a low expression of EGFR, but a high expression of ERBB2. According to this immunohistochemistry-based classification, genetically unstable tumours had a worse DSS compared to urobasal tumours, but better than squamous cancer cell-like tumours in a mixed population of NMIBC and MIBC[27]. Recent work from our own group has also shown high *CDKN2A* expression to be associated with shorter progress-free survival in T1 urothelial carcinoma[30]. The group of *CDKN2A*^{high} MIBC consistently identified in a proportion between 10.0 and 12.6% in all three datasets analyzed in the present study therefore might reflect a subgroup of genomically instable tumours, which account for 21.5% of advanced bladder cancers as described by Sjödahl *et al.*[29]. The reported overexpression of *CDKN2A* in genomically unstable tumours could be a sign of an in vain countermeasure to reduce cell cycle activity, which is deregulated due to other molecular aberrations.

Controversial to an association with the genomically unstable subtype is the fact, that 71,7% of the tumours with *CDKN2A*^{high} from the TCGA cohort are termed as basal squamous according to the 2017 TCGA publication[19]. Yet, this group (35% of MIBC in the TCGA cohort), also comprises 41% of tumors with *CDKN2A* deep deletions, meaning that *CDKN2A*^{high} tumours, which do not show any homozygous deletion of *CDKN2A*, are in part not a representative, but a highly selected subgroup of basal squamous tumours. In line with this and unlike reported for basal squamous tumors in the TCGA publication, *CDKN2A*^{high} tumours also do not show an elevation of *PDCD1*, *CD274* and *CTLA4* expression.

With regard to drug target gene expression, *CDKN2A* expression showed a negative correlation with *FGFR3* expression in the TCGA cohort and a trend towards a negative correlation in the test cohort. The TCGA publication from 2014 proposed a correlation between *CDKN2A* deletion or underexpression and activating mutations of *FGFR3* or *FGFR3* overexpression[4]. They also reported an inverse correlation between *CDKN2A* and *FGFR3* RNA expression. According to their mutational data they proposed three subtypes of bladder cancer: (A) *focally amplified*, (B) *papillary CDKN2A-deficient and FGFR3-mutant* and (C) *TP53/cell-cycle-mutant*. The study of Rebouissou and colleagues confirmed a high incidence of *CDKN2A* deletions (hemizygous 23.7%, homozygous 17.5%) and *FGFR3* mutations (62.1%) in NMIBC[15]. In MIBC the rates of *CDKN2A* deletions were even higher (hemizygous 27.9%, homozygous 22.5%). Both in NMIBC and in MIBC there was a significant coincidence of *CDKN2A* deletions and activating mutations of *FGFR3* and NMIBC tumours with this feature had an increased progression rate.

Due to their low *FGFR3* expression, patients with CDKN2A^{high} tumours presumably do not seem to be suitable candidates for a therapy targeting FGFR3, whilst patients with a high *FGFR3* expression might benefit from such an approach. The tyrosine kinases inhibitor Pazopanib is already approved for the treatment of advanced or metastatic kidney cancer and certain sarcoma entities, but there is only limited data about its application in MIBC: A small phase II trial on 19 unselected patients with metastatic bladder cancer reported a median PFS of only 1.9 months[31]. Another phase II study of 41 unselected patients with metastatic bladder cancer after failure of chemotherapy reported an overall initial response rate of 17%, but PFS and OS were poor[32]. However, there were also two patients with sustained long-term response. The RNA expression of *FGFR3* in these tumours is not reported in the trial. Another group reported a case of a woman with a metastatic bladder cancer carrying an activating *FGFR3* mutation[33]. This patient showed a durable remission of more than 6 months upon treatment with Pazopanib. *In vitro* results also suggest a synergistic effect of Pazopanib with Docetaxel in the treatment of bladder cancer cells[34], pointing to a potential role of Pazopanib in combination therapy of cases with a suitable molecular

profile. Besides Pazopanib, several other substances targeting FGFRs are currently under investigation[35] and AZ12908010, AZD4547, PD173074, TKI-258/Dovitinib, SU5402 and BGJ-398 showed promising results *in vitro*[36–39]. Yet, clinical data is scarce and partially controversial: By systemic administration of Dovitinib biologically active concentrations could be consistently achieved in 13 patients with NMIBC[40]. However, long-term administration was not possible due to frequent toxicities. In another study Dovitinib showed a better tolerability but the antineoplastic effect in patients with FGFR3-mutated and FGFR3 wild type urothelial bladder cancer was poor[41]. For AZD4547 a case of long term response is described[42]. BGJ-398 showed an overall response rate of 36% in patients with pretreated advanced or metastatic urothelial carcinoma and was well tolerated[43].

Besides a low *FGFR3* expression, *CDKN2A*^{high} tumours also showed a low *ESR2* expression and *AR* was negatively correlated with *CDKN2A*. *CDKN2A* expression was positively correlated with *PDCD1*, *CD274* and *CTLA4* expression. Yet, this did not result in a differential expression in *CDKN2A*^{high} tumours. In general the expression of the tested drug target genes was rather low in *CDKN2A*^{high} tumours. Therefore they may represent a high risk population both in terms of prognosis and limited treatment options.

Correlation of *CDKN2A* with the downstream markers *CDK4*, *RB1* and *E2F3* in the TCGA cohort also point to a more complex role of *CDKN2A* in the biology of MIBC: Unlike to be assumed by the known mechanism of *CDKN2A*-*CDK4* interaction, with *CDKN2A* typically deactivating *CDK4*, the expression of both genes is not inversely but positively correlated. Furthermore, the tumour suppressor *RB1*, which is the subsequent gene in this signaling cascade[44], is significantly downregulated upon increasing *CDKN2A* expression, indicating a more active cell cycle despite a high *CDKN2A* expression. Since the P16 protein mainly functionally regulates downstream targets via binding of CDK4 and CDK6, preventing them from interaction with cyclin D, which then results in reduced phosphorylation of RB1, phosphorylation data of RB1 would offer a more precise information about the pathway activity downstream of P16. Yet, there are currently no larger datasets analyzing RB1 phosphorylation status in bladder cancer.

The 2014 TCGA publication, comprising data from 131 MIBC, described four different subtypes based on RNA expression data[4]. These subtypes are mainly determined by the expression of luminal cytokeratins *KRT8* and *KRT18*, basal cytokeratins *KRT5*, *KRT6A*, *KRT6B*, *KRT6C*, *KRT15*, the transcription factors *GATA3* and *FOXA1* and uroplakins. The updated 2017 publication[19] suggests five molecular subtypes and implemented elements from other subtyping approaches[8,9,45]. We correlated the expression of *CDKN2A* with the aforementioned subtype determining genes. In the TCGA cohort there was a negative correlation with *GATA3* and *FOXA1* ($\rho = -0.162$ and -0.299), which are urothelial differentiation markers. For both genes also a significantly lower expression was seen in *CDKN2A*^{high} tumours. And also *KRT20*, typically associated with luminal tumours, showed a lower expression in this group. Fitting to this TCGA basal squamous tumours have the highest *CDKN2A* expression, whilst tumours from the luminal TCGA subtype group had an exclusively low expression.

Our results are limited by the fact that both our *CDKN2A* PCR assay and the RNA expression analysis in the MDA and the TCGA cohort do not discriminate between the *CDKN2A* transcripts. Therefore further analyses substratifying between the transcripts for p16 and p14 could add valuable information.

Furthermore the three analyzed cohorts are not directly comparable due to the different techniques (qRT-PCR in the test cohort, RNA expression microarray in the MDA cohort and RNAseq in the TCGA cohort) used for quantification and different data normalization protocols.

Taken together the *CDKN2A* RNA expression does not present as a continuum. On the one hand this indicates that quantifiably tools can be helpful to accurately deduce prognosis from *CDKN2A* RNA expression. On the other hand this also reflects a more complex biology behind the variation of *CDKN2A* expression in MIBC. In line with this, the expression of *CDKN2A* and p16 differs among molecular subtypes.

With the chosen approach we were able to identify a subgroup of patients with high *CDKN2A* expression with poor prognosis and comparably low expression of drug target genes. These tumours seem to partly correspond to both the basal squamous subtype described in the 2017 TCGA classification[19] and the genomically unstable tumours described by Sjödahl *et al.*[29]. *CDKN2A* therefore might be a valuable component in the molecular risk stratification of MIBC and a potential indicator for targeted therapy decision-making.

Methods

Study population, RNA isolation and qRT-PCR. The test cohort consisted of 80 patients (mean age 66 years, range 46–93 years) with MIBC who underwent radical cystectomy at the Mannheim University Hospital Center between January 1998 and December 2006. Clinical and pathological data were retrospectively obtained from medical records (ethics approval 2016-814R-MA of the medical ethics committee II of the medical faculty Mannheim of the University of Heidelberg).

RNA extraction was performed as described before[46]. 10 µm sections from FFPE tissue samples were used for RNA extraction with a commercially available bead-based extraction method (XTRACT kit; STRATIFYER Molecular Pathology GmbH, Cologne, Germany). RNA was eluted with 100 µl elution buffer and stored at −80 °C until used.

The RNA expression of *CDKN2A* and *FGFR3* was determined in relation to the housekeeping gene calmodulin 2 (*CALM2*) using 1-step qRT-PCR with validated TaqMan gene expression assays (STRATIFYER catalogue numbers: *CDKN2A*: MP672, *FGFR3*: MP599 and *CALM2*: MP501). Primers and labeled hydrolysis probes were selected using Primer Express® Software (Applied Biosystems/Life Technologies, Karlsruhe, Germany) and were controlled for single nucleotide polymorphisms. All primers, probes and amplicons were checked for their specificity against nucleotide databases at NCBI using Basic Local Alignment Search Tool (BLAST). Primers and probes were purchased from Eurogentec S.A. (Seraing, Belgium). For each primer/probe set, the amplification efficiency was tested, aiming to reach comparable efficiency of >90% (efficiency range from 97.7 to 99.7%). Primers and hydrolysis probes were diluted to 100 µM, using a stock solution with nuclease-free water (Life Technologies

GmbH, Darmstadt, Germany). qRT-PCR was applied for the relative quantification of *CDKN2A* and *FGFR3*. For PCR, 0.5 µM of each primer and 0.25 µM of each probe were used. All quantitative reverse-transcription PCRs were performed in triplicates using the SuperScript® III Platinum® One-Step qRT-PCR kit (Invitrogen/Life Technologies, Darmstadt, Germany) according to the manufacturer's instructions. Experiments were performed on a Stratagene Mx3005p (Agilent Technologies, Waldbronn, Germany) with 30 min at 50 °C and 2 min at 95 °C followed by 40 cycles of 15 s at 95 °C and 30 s at 60 °C. PCR amplification of each gene was performed in triplicates in each patient. Expression relative to CALM2 was determined using the 40-ΔCT method.

Reanalysis of existing datasets. 57 patients with MIBC (mean age 66 years, range 41–89) from the MDA cohort[45] and 365 patients with MIBC (mean age 68 years, range 34–90 years) identified from the TCGA (The Cancer Genome Atlas) project served for outcome validation. Illumina array RNA expression data of the MDA cohort was downloaded from Gene Expression Omnibus (GSE48276).

TCGA RNA sequencing expression data (z-score normalized data) of *CDKN2A* and the drug target genes *FGFR3*, *AR*, *ESR1*, *ESR2*, *ERBB2*, *PDCD1* (PD1), *CD274* (PDL1) and *CTLA4* were downloaded from CBioPortal[47]. Furthermore the expression of TCGA molecular subtype determining genes *KRT5*, *KRT6A*, *KRT6B*, *KRT6C*, *KRT8*, *KRT14*, *KRT18*, *KRT20*, *UPK1A*, *UPK1B*, *UPK2*, *UPK3A*, *UPK3B*, *GATA3* and *FOXA1*, of the *CDKN2A* downstream target genes *CDK4 and RB1*, the copy number variation (CNV) data of *CDKN2A* and the annotated updated TCGA RNA expression subtypes[19] were extracted.

Statistics. Statistical analyses were performed using SAS JMP version 11.0 (SAS Institute, Cary, NC, USA) and Graphpad PRISM (Version 7.0; Graph Pad Software Inc., La Jolla, CA, USA). Cut-Off definitions were done by partitioning tests for decision trees to determine different *CDKN2A* expression groups. Student's t-test and Chi^2 test were used to compare for differences in the distribution of clinical parameters, TCGA subtypes and *CDKN2A* CNV data between the *CDKN2A* expression groups.

Kaplan Meier analyses were performed for DSS and RFS in the test cohort and for DSS and OS in the validation cohorts and were tested for significance using Log-Rank test.

Both in the test cohort and in the TCGA cohort *CDKN2A* expression was correlated with *FGFR3* expression using Spearman correlation. In the TCGA cohort *CDKN2A* expression was also correlated with the expression of *AR*, *ESR1*, *ESR2*, *ERBB2*, *PDCD1*, *CD274*, *CTLA4*, *KRT5*, *KRT6A*, *KRT6B*, *KRT6C*, *KRT8*, *KRT14*, *KRT18*, *KRT20*, *UPK1A*, *UPK1B*, *UPK2*, *UPK3A*, *UPK3B*, *GATA3*, *FOXA1*, *CDK4, RB1, E2F2* and *KI67*.

Student's t-test was used to test for differences in gene expression in the *CDKN2A* expression groups in the test cohort and the TCGA cohort. For the analysis of *CDKN2A* expression according to *CDKN2A* copy number status and RNA-expression subtypes Kruskal-Wallis test with post hoc Dunn's test for multiple comparisons was performed in the TCGA cohort. Graphs were designed with Graphpad Prism. P-values < 0.05 were deemed statistically significant.

Ethical approval. All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Due to its retrospective character, for this type of study formal consent is not required.

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Author Contributions

R.S., W.O., A.H., C.B., R.M.W. and P.E. planned the study and the experiments. T.S.W., C.A.W. and P.E. assembled the test cohort. C.A.W., S.B., R.S., M.E. and A.H. performed pathologic evaluation of tissue sections. R.M.W. performed PCR analyses. T.S.W., A.H., R.M.W. and P.E. performed PCR data analysis. T.S.W., A.H., R.M.W. and P.E. performed analyses of the TCGA cohort. T.S.W., J.B., A.H. and P.E. wrote the manuscript. All authors reviewed the manuscript.

Additional Information

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