Introduction

Treatment of patients with metastatic colorectal cancer (mCRC) is challenged by high incidence and poor patient outcome (1). Chemotherapy has been the standard approach for decades and within the last 10 years targeted therapies have been added. Modulation of angiogenesis, the development of new blood vessels from the preexisting vasculature, constitutes one of the main efforts in this context. A monoclonal antibody targeting vascular endothelial growth factor A (VEGF-A) was the first antiangiogenic drug to be approved in 2004 (Avastin; bevacizumab). Following this came the recently approved afibbercept, a fusion protein (VEGF "trap") targeting a broader spectrum of the VEGF-related ligands, and regorafenib, a multitargeted tyrosine kinase inhibitor. Additional drugs targeting the angiopoietins Ang-1 and -2, and the placental growth factor are currently being tested in clinical trials. A parallel identification of predictive biomarkers is, however, a prerequisite for the rational implementation of these drugs.

Growing evidence calls for the attention toward epidermal growth factor–like domain 7 (EGFL7) and microRNA-126 (miR126) as key regulators of the angiogenic endothelial growth factor-A and EGFL7 axis in this setting. Mol Cancer Ther; 13(9); 2238–45. ©2014 AACR.
scoring technique. If confirmed, the results may gain clinical interest focusing on the current testing of anti-EGFL7 in combination with chemotherapy and anti-VEGF-A in patients with mCRC (CONGO trial).

We have previously shown that miR126 expression in the primary tumor, visualized by in situ hybridization (ISH) and quantified by image-guided analyses, is predictive of chemotherapy efficacy (10). In addition, we recently demonstrated that miR126, quantified by RT-qPCR, was not associated with clinical response to chemotherapy combined with anti-VEGF-A (9). In the initial chemotherapy-only study (10), we hypothesized that the low RR seen in patients with low miR126 expression was caused by a higher degree of immature blood vessels with low integrity. Tumor-associated angiogenesis, dominated by immature and leaky blood vessels, is known to be highly dependent on VEGF-A as an EC survival factor (11–13), and patients with low miR126 expression may consequently benefit the most from the addition of anti-VEGF-A. A possible prognostic value of miR126 in mCRC has been proposed as well (9, 10, 14).

Whether the clinical impact of these biomarkers is the same, if analyzed in tumor resections or endoscopic biopsies, remains to be elucidated.

Consequently, with the aim of clarifying the clinical impact of EGFL7 and miR126 as predictive biomarkers in a first-line setting of chemotherapy and anti-VEGF-A, we designed this translational study based on EGFL7 immunohistochemistry (IHC) and miR126 ISH. Both parameters were quantified by image-guided analysis, in resections as well as endoscopic biopsies from primary tumors of patients with mCRC.

Patients and Methods
Reporting in this study is in accordance with the REMARK (15) and BRISQUE (16) criteria.

Patient characteristics
The translational study included two comparable cohorts of patients with histologically verified mCRC treated with first-line therapy (Table 1). Cohort 1 consisted of 86 patients treated with capcitabine and oxaliplatin (XELOX) plus bevacizumab at the Department of Oncology, Vejle Hospital (Vejle, Denmark) between March 2008 and August 2012. Cohort 2 comprised 72 patients treated with induction chemotherapy consisting of fluorouracil and leucovorin or capecitabine in combination with either oxaliplatin (FOLFOX or XELOX) or irinotecan (FOLFIRI or XELIRI) plus bevacizumab between October 2007 and 2009 (phase III Nordic ACT trial, patients treated at Danish centers only; ref. 17). Forty-five patients (from cohort 2) without progressive disease, planned surgery, or unacceptable toxicity, were subsequently randomized to maintain bevacizumab ± erlotinib [anti-epidermal growth factor receptor (EGFR)] until progression or unacceptable toxicity. Adjuvant treatment, if given, was completed at least 6 months before initiation of first-line therapy in both cohorts. None of the patients in the two cohorts had received neoadjuvant therapy.

Clinical data were recorded according to good clinical practice. The study was approved by the Regional Scientific Ethical Committee of Southern Denmark and the Danish Data Protection Agency (cohort 1, S-20080104 and S-20100005; cohort 2, VF-20060115, 2007–41–0252). Written informed consent was obtained from all patients enrolled in the study.

Sampling
Available biospecimens consisted of 97 endoscopic biopsies from the primary tumor and 85 resected primary tumors. Among these samples, paired endoscopic biopsies and tumor resections from the same patient were available in 24 cases. Some endoscopic biopsies and resections contained limited tumor tissue allowing only for the EGFL7 or miR126 analysis. Sample distribution is specified in Fig. 1.

All histologic samples followed routine formaldehyde fixation and paraffin embedding (FFPE) and were stored and transported at room temperature. The median storage time from sampling to analysis was 3.7 years. All FFPE tissue blocks were processed at the Department of Pathology, Vejle Hospital. Tissue sections for EGFL7 IHC and miR126 ISH analyses were cut adjacent from the same FFPE tissue block.

Evaluation of treatment efficacy
RRs were assessed according to the Response Evaluation Criteria in Solid Tumors (RECIST 1.0) (18). A total of 150 patients was available for response evaluation. Four patients were censored from response evaluation because they only received one or two cycles of therapy, 3 patients did not have evaluable lesions according to the RECIST criteria, and one patient changed to second-line therapy before progression (Fig. 1). Evaluations were based on clinical examination and CT scans of the chest and abdomen. Responding patients were classified as having either complete response or partial response, whereas nonresponding patients were classified as stable disease or progressive disease.

EGFL7 immunostaining
Tissue sections were stained with an antibody against EGFL7 as previously described (9). Four-micrometers-thick tissue sections were mounted on coated slides and dried for half an hour at 60°C and then overnight at 37°C. Deparaffinization was performed in xylene for 10 minutes at room temperature followed by rehydration in graded alcohol solutions (99%–70%). Endogenous peroxidase was blocked by adding hydrogen peroxide 3% for 5 minutes. Antigens were demasked by heat-induced epitope retrieval in a microwave oven using a TEG buffer (TRIS 10 mmol/L, EGTA 0.5 mmol/L, and Titriplex-VI) at pH 9 for 10 minutes at 1000 W and 15 minutes at 440 W. Tris-buffered saline (TBS)/Tween pH 7.6 was added for 5 minutes after cooling at room temperature. The
anti-EGFL7 was a rabbit polyclonal antibody (ab115786; Abcam) used in a 1:200 dilution (diluted in Dako REAL Antibody Diluent; S2022) and incubated for 90 minutes (room temperature). After washing in TBS/Tween, the visualization was performed using Dako EnVision ™ System-HRP (DAB) for rabbit primary antibodies, K4011 for 30 minutes. All sections were counter stained with Mayer hematoxylin.

The specificity of the anti-EGFL7 antibody was tested using 4-mL EGFL7 recombinant protein, Novus Biologicals H00051162-P01 and 1 mL of the anti-EGFL7 antibody, in 200 mL Dako EnVision FLEX WASH BUFFER K8007 (2.4 mmol/L protein vs. 0.6 mmol/L antibody). The solution was preincubated for 30 minutes followed by 90 minutes of incubation on the tissue sections (room temperature). This resulted in dramatically reduced staining of ECs, demonstrating only a faint staining reaction, and abolished background staining and staining of tumor cells was observed.

**miR126 in situ hybridization**

The miR126 ISH was performed at Bioneer on an automated platform as previously described (19, 20). In brief, 6-μm-thick tissue sections were pretreated with a proteinase-K solution followed by hybridization with a double 6-carboxyfluorescein (FAM)-labeled locked nucleic acid (LNA) miRCURY probe (LNA microRNA detection probe; Exiqon A/S) specific for human miR126.
stringent washes in saline sodium citrate (SSC) buffers, the probes were detected with alkaline phosphatase-conjugated sheep anti-FAM Fab fragments (Roche). Of note, 4-nitro-blue tetrazolium and 5-brom-4-chloro-3-indolyl-phosphate substrate (Roche) were added and samples were incubated leading to a blue precipitate (the ISH signal). Counterstaining was performed with nuclear fast red. All slides were processed in a Tecan Freedom Evo automated hybridization instrument (Tecan; ref. 20) in series of 48 slides per run.

**EGFL7 and miR126 image analyses**

**EGFL7.** The Visiopharm integrated microscope and software module (Visiopharm) were used for image analysis. An overview image was obtained for each slide using a ×1.5 objective. The tumor area was encircled to obtain ×20 (∼282 total magnification on the computer monitor) images for quantification of EGFL7. Two image acquisition configurations were applied depending on whether the samples were whole-tumor resections or endoscopic biopsies. For tumor resections, up to 25 nonoverlapping, randomly positioned ×20 images per sample were collected. All images were within the tumor tissue compartment. For endoscopic biopsies, ×20 images were collected covering the whole tissue area. The pixel classifier prepared for the tumor resections generated estimates that were 11% higher than the estimates obtained with the endoscopic biopsy–specific classifier, probably due to the increased area contribution from empty glands associated with the small biopsies. These estimates were consequently corrected for with a factor 1.11. To allow direct comparison of the EGFL7 estimates from resection samples and biopsy samples, all estimates were calculated as [area of stained ECs] × [1.11]/[total tissue area detected], in which total tissue area detected also included nuclei and connective tissue within the invasive tumor area as mean values of ×20 images for each patient. Thus, the EGFL7 estimates are presented as area fractions without a dimension. Strong DAB staining was only seen in EC, whereas sporadic DAB staining was detected particularly in smooth muscle and would, therefore, give a minor contribution to the estimate. The classifier was, thus, adjusted to count only the strong DAB signal providing an EC-specific EGFL7 estimate for the analyses expressed as vessel area (VA). The staining of the endoscopic biopsies resulted in expression of higher background levels, meaning that more noise would be included in these quantitative estimates despite the adjustment of the classifier. Some samples showed widespread EGFL7 staining, causing very high area fractions (>20), and, thus, the data obtained from the endoscopic biopsies and tumor resections were analyzed and presented separately. Images with tumor ulceration and necrotic tissue, staining artifacts, and no cancer cells, were excluded.

**miR126.** Image acquisition was achieved using the same principles as described for EGFL7 with a few exceptions. The color classifier was prepared to determine the area fraction represented by the blue miR126 ISH signal (expressed as VA). The mean score from the sampled images was calculated for each patient as described above for EGFL7. The image analysis protocols were performed by one observer, Boye Schnack Nielsen, unaware of the clinical outcome.

**Statistical analysis**

Median values were compared using the Wilcoxon rank-sum test. The Fisher exact test was used for comparison between categorical parameters. Linear regression analysis was used to investigate the linear association between continuous variables. The progression-free survival (PFS) was defined as the time from start of treatment until the first documented tumor progression or death of any cause. The PFS data were censored in case of metastasectomy or radio frequency ablation of metastasis during the course of first-line treatment (n = 19), in case of initiation of second-line therapy before documented progression (n = 1), and in case of early withdrawal (n = 4). Such data were censored from the day of the event. The log-rank test was used to test for differences between the groups in the survival functions and the results were illustrated by the Kaplan–Meier method. All statistical calculations were carried out using the NCSS statistical software (NCSS Statistical Software; version 2007). P values <0.05 were considered significant, and all tests were two-sided.

**Results**

A signal from the EGFL7 IHC analysis was present in all samples, demonstrating intense immunoreactivity in ECs (Fig. 2A) and only sporadic, faint staining in smooth muscle cells, adipocytes, myofibroblasts, and cancer cells. The miR126 ISH analysis resulted in a specific visualization of ECs in all tumor resections as well as in the endoscopic biopsies (Fig. 2B).

**Patient characteristics**

Patient follow-up ended July 1, 2013. The median follow-up period was 20 months. Patient characteristics are shown in Table 1. The only significant difference between
the two patient cohorts was the surgical intervention. The higher percentage of the patients from cohort 2 with primary tumor resection performed before therapy was higher than that of cohort 1. Despite this difference, the clinical endpoints, RR, and PFS of the cohorts were comparable justifying analyses on the pooled dataset.

The EGFL7 VA in tumor resections was significantly associated with the \( \text{KRAS} \) mutation status. Patients with \( \text{KRAS} \)-mutated tumors presented with a median EGFL7 VA of 8 [95% confidence interval (CI), 6–9], whereas patients with \( \text{KRAS} \) wild-type tumors had a median score of 4 (95% CI, 3–9), \( P = 0.049 \). No significant associations were demonstrated between the miR126 VA and the clinicopathologic characteristics as shown in Table 1 (\( P > 0.05 \)).

**Correlations**

The four correlation analyses between EGFL7 and miR126 VA in endoscopic biopsies and tumor resections are illustrated in Fig. 3. Neither the EGFL7 nor the miR126 VA in the endoscopic biopsies was correlated with the corresponding scores in the tumor resections, although the EGFL7 correlation demonstrated a borderline significance (\( r = 0.40, P = 0.06 \)). Positive significant correlations were demonstrated between the EGFL7 and miR126 VA in tumor resections as well as in biopsies (\( r = 0.33, P = 0.002 \) and \( r = 0.54, P < 0.0001 \), respectively).

**Treatment efficacy**

The relationship between RR and EGFL7 and miR126 VA in endoscopic biopsies and tumor resections is demonstrated in Supplementary Table S1. The EGFL7 VA in tumor resections was the only parameter significantly related to treatment response with a median EGFL7 VA in responding patients of 4 (95% CI, 4–6) compared with 8.5 (95% CI, 7–11) in nonresponders, \( P = 0.0008 \). The distribution of EGFL7 VA according to the four response groups is illustrated in Fig. 4. Addressing the cohorts separately the relationship between RR and EGFL7 VA remained significant in both cohorts, thereby demonstrating in-study validation (\( P = 0.0007 \) and \( P = 0.03 \)).

The predictive value of EGFL7 VA in tumor sections to treatment response to first-line chemotherapy combined with anti–VEGF-A was analyzed using the median EGFL7 VA in responding patients as cutoff (4.0). This strategy resulted in a positive predictive value of 20 of 27 (74%) and a negative predictive value of 35 of 52 (67%).

The relationship between EGFL7 and miR126 VA in tumor resections in regard to PFS is illustrated in Fig. 5A and B. Neither the EGFL7 VA nor the miR126 VA resulted in obvious differences in relation to PFS, although borderline significance was detected for EGFL7 VA (\( P = 0.06 \)).

**Discussion**

The identification of patients likely to respond to antiangiogenic treatment has high priority. In this translational study, we analyzed the predictive value of the angiogenic couple, EGFL7 and miR126, in primary tumors from patients with mCRC treated with first-line chemotherapy and bevacizumab. The results indicated that the EGFL7 VA has a predictive value in this setting.

We analyzed two cohorts of patients with mCRC. The only difference between the two investigated cohorts was
a higher fraction of patients with resected tumors compared with endoscopic biopsies in cohort 2. This difference, however, did not influence any of the clinical endpoints and, thus, allowed us to consider the two patient cohorts as one when analyzing the investigated parameters.

The present results demonstrate a significant difference in median EGFL7 VA in the tumor resections according to RR. Patients with low EGFL7 VA were more likely to respond to chemotherapy combined with anti–VEGF-A. With these findings, we corroborate the borderline significant relationship detected in our recent study, in which EGFL7 expression was scored manually using a point-counting graticule (9). Furthermore, analysis of the two cohorts allowed in-study validation and a similar significant difference could be detected in the two distinct cohorts. The results implied a numerical difference between EGFL7 VA in the two cohorts; however, no significant differences were detected in a following subgroup analysis (data not shown). The lack of relationship between RR and EGFL7 VA in the biopsies may very well be explained by methodologic reasons as discussed below.

EGFL7 is also upregulated under pathologic angiogenesis and is believed to serve as an anchoring protein connecting the immature blood vessels in the sprouts to the extracellular matrix, thereby supporting the angiogenic process (3, 21). High EGFL7 expression has been associated with increased tumor growth partly mediated by preventing invasion of immune cells into the tumor (2). These previous findings taken together support the plausibility of our current results demonstrating increased efficacy of an anti–VEGF-A regimen in tumors with low EGFL7 VA, and draws attention to the current testing of an anti-EGFL7 antibody in combination with chemotherapy plus anti–VEGF-A in patients with mCRC (CONGO trial).

One of the aims of the present study was to elucidate whether analyses of the investigated parameters in tumor tissue from an endoscopic biopsy would be feasible. The analytic phase indicated differences between the assay readouts from resections and biopsies in relation to both EGFL7 and miR126, which led to a higher degree of background staining/noise to be counted in the endoscopic biopsies. These results, along with the fact that the EGFL7 and miR126 VA in the
Paired biopsies and resections (Fig. 3) were not significantly correlated, motivated us to focus primarily on the resections in the following comparisons with the clinical endpoints. The positive correlation between the EGFL7 and miR126 VA in the biopsies (Fig. 3) indicates that analyzing EGFL7 and miR126 VA in biopsies provides reliable estimates justifying this approach in other settings in which whole-tumor/metastasis resections may not be available.

When comparing EGFL7 and miR126 VA (adjacent sections), we detected a positive, significant relationship in biopsies and tumor resections, respectively, indicating some degree of common transcriptional regulation of miR126 and EGFL7. This is not surprising, because transcription of the EGFL7 gene also involves transcription of miR126 (22–24).

Another interesting finding in this study was a significant relationship between KRAS mutational status and EGFL7 VA in the tumor resections. The KRAS-mutated tumors presented with a higher median EGFL7 VA. Although the KRAS mutational status has previously been associated with modulation of angiogenesis in colorectal cancer (25), we were unable to identify a direct link between KRAS and EGFL7 in the literature. It is, however, likely that constitutively activated KRAS signaling may influence the expression of the EGFL7 gene. The lack of association with the miR126 VA suggests differential regulation of the translation of EGFL7 relative to the level of miR126. The possible clinical importance of this observation needs further investigation.

The lack of relationship between miR126 VA and RR in this study corresponds to our hypothesis drawn from our chemotherapy-only study (10), in which low miR126 VA, and thereby low blood vessel integrity was associated with decreased RR. Such patients were thought to benefit the most from anti-VEGF-A therapy, and although representing an indirect conclusion, the disadvantage of low miR126 expression detected in our previous study may have been neutralized by the treatment with anti-VEGF-A in this study.

The relationship between EGFL7 VA in the tumor resections and RR translated into a borderline significant difference in PFS favoring patients with low EGFL7 VA. This sample size may explain this result and it is likely that a similar analysis in a larger cohort would have resulted in a significant separation of the PFS Kaplan–Meier curves. The arbitrary choice of the median as cutoff may be an additional explanation. miR126 VA in the tumor resections did not show any sign of relationship with PFS as expected on the basis of the results about RR. This, however, differs from our previous study in which high miR126 was associated with a favorable prognosis (9). The reasons may be differences in the tissue analyzed (biopsies vs. resections) and methods applied (qPCR vs. ISH).

This study provides the first significant results, from two independent cohorts, arguing for a predictive value of EGFL7 VA, in regard to first-line chemotherapy combined with bevacizumab in patients with mCRC. These results strengthen the candidature of an anti–EGFL7-targeted approach. The possible role of KRAS in this setting calls for further investigations.
Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Disclaimer
None of the funders had any influence on any part of the study.

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References
Epidermal Growth Factor–like Domain 7 Predicts Response to First-Line Chemotherapy and Bevacizumab in Patients with Metastatic Colorectal Cancer

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