Tumor Phosphatidylinositol-3-Kinase Signaling and Development of Metastatic Disease in Locally Advanced Rectal Cancer

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Abstract

Background: Recognizing EGFR as key orchestrator of the metastatic process in colorectal cancer, but also the substantial heterogeneity of responses to anti-EGFR therapy, we examined the pattern of composite tumor kinase activities governed by EGFR-mediated signaling that might be implicated in development of metastatic disease.

Patients and Methods: Point mutations in KRAS, BRAF, and PIK3CA and ERBB2 amplification were determined in primary tumors from 63 patients with locally advanced rectal cancer scheduled for radical treatment. Using peptide arrays with tyrosine kinase substrates, ex vivo phosphopeptide profiles were generated from the same baseline tumor samples and correlated to metastasis-free survival.

Results: Unsupervised clustering analysis of the resulting phosphorylation of 102 array substrates defined two tumor classes, both consisting of cases with and without KRAS/BRAF mutations. The smaller cluster group of patients, with tumors generating high ex vivo phosphorylation of phosphatidylinositol-3-kinase-related substrates, had a particularly aggressive disease course, with almost a half of patients developing metastatic disease within one year of follow-up.

Conclusion: High phosphatidylinositol-3-kinase-mediated signaling activity of the primary tumor, rather than KRAS/BRAF mutation status, was identified as a hallmark of poor metastasis-free survival in patients with locally advanced rectal cancer undergoing radical treatment of the pelvic cavity.

Introduction

The multitude of more than 500 protein kinases, the kinome, represents a substantial part of the human genome, and receptor tyrosine kinases are key mediators in signaling cascades regulating central biological processes of malignancy, such as proliferation, angiogenesis, and metastasis [1,2]. In order to optimize and individualize therapeutic efficacy of kinase inhibiting agents for metastatic disease control, it seems rational to exploit the specific pattern of tumor kinase activity as functional biomarker of actionable targets.

In locally advanced rectal cancer (LARC), randomized studies will develop metastatic disease as result of early, undetected systemic dissemination of tumor cells. Within this frame of reference, our prospective non-randomized study comprising LARC patients given CRT followed by radical surgery and no further treatment offers a unique opportunity to explore the regulatory role of specific kinase signaling pathways in tumor proliferation, angiogenesis, and metastasis in a defined clinical context. In this study, using peptide arrays with tyrosine kinase substrates [4–6] to analyze the patients’ tumors at the time of diagnosis, we have found that patients with poor CRT response had significantly elevated tumor kinase activity, representing signaling mediated by VEGFR, EGFR, and phosphatidylinositol-3-kinase (PI3K)/AKT, compared to good-responding patients [7]. Moreover, we have reported that tumor angiogenic signatures comprising PDGFR, VEGFR, and EPOR were associated with microscopic dissemination of tumor cells in bone marrow at the
time of diagnosis, which secondly was correlated with heightened risk of developing metastatic disease following the course of radical treatment of the pelvic cavity [8].

In metastatic colorectal cancer, monoclonal antibodies directed against EGFR, currently cetuximab and panitumumab, have been implemented in clinical practice for the last eight years. For the optimum selection of eligible patients, initial molecular data established mutations of genes encoding effector proteins downstream of EGFR in the tumor signaling cascade; primarily mutations in codon 12 or 13 of KRAS, as predictor of intrinsic therapeutic resistance to anti-EGFR monoclonal antibodies [9]. Moreover, mutations in genes encoding other mediators, primarily BRAF, pV600E but also PIK3CA mutations, are associated with resistance [9], while tumors harboring KRAS p.G13D may respond [10,11]. It was recently suggested that amplification of ERBB2 comprises another resistance mechanism [12,13], and that [10,11].

Hypothesizing that kinase signaling activity conducted by EGFR may reflect mutation status of genes encoding effector proteins from any component of the molecular network, we compared the previously achieved ex vivo tumor phosphopeptide profiles from the LARC study patients [7,8] with tumor mutations within KRAS exon 2, BRAF exon 15, and PIK3CA exons 9 and 20, and amplification of ERBB2. Conceptually, tumor kinase activity signatures comprising all interacting signaling pathways of relevance might be developed into functional biomarkers of actionable therapy targets for metastatic disease control. The investigations in this study defined a subgroup of LARC patients, following the resection of primary tumors with high activity of the PI3K signaling pathway, with particularly poor metastasis-free survival. This finding suggests that high tumor PI3K-mediated signaling activity is a biomarker of risk assessment and treatment stratification.

Materials and Methods

Ethics Statement

The phase II, non-randomized study protocol (ClinicalTrials ID NCT00278694) was approved by the Institutional Review Board and the Regional Committee for Medical and Health Research Ethics of South-East Norway, and is in accordance with the Helsinki Declaration. Written informed consent was required for participation.

Patients and Procedures

The patient population reported here was enrolled between October 2005 and May 2008. Patient eligibility criteria, evaluation procedures, study treatment, and review procedures of follow-up have been described in detail previously [7]. Following neoadjuvant fluoropyrimidine−/−oxaliplatin-based CRT and subsequent surgery, the resected primary tumor specimens were histologically evaluated for treatment response according to standard criteria (histopathologic staging; ypTN) and histomorphologic tumor regression grade (TRG). Briefly, the latter was graded within one of five TRG categories, spanning from the absence of residual tumor cells in the resected specimen (TRG1) to the lack of morphologic signs of treatment response (TRG5) [21]. Follow-up data was obtained from the clinical database and censored on December 31, 2011. Valid observations of the presence or absence of distant metastases required designated radiologic examination. Four patients with synchronous resectable liver metastases were excluded from analysis of metastasis-free survival.

Tumor Samples

At the time of diagnosis, baseline study-specific primary tumor biopsies were obtained from 79 patients with locally advanced rectal cancer under heavy sedation, snap-frozen in liquid nitrogen, and stored at −80 °C, as reported previously [7]. Of the included patients, 16 patients were excluded from the present study, as 12 patients had tumor biopsy specimens in which kinase activity profiling had not been performed because the patients were either ineligible after study registration (n = 4), had withdrawn consent (n = 1), had unexpectedly died during the preoperative treatment (n = 1), had developed metastatic disease progression during preoperative treatment that precluded definitive surgery (n = 1), had tumor cell content less than 20% within the biopsy specimen (n = 3), or had a biopsy specimen in which kinase activity analysis was missing of unknown reasons (n = 2), and four additional patients did not have tumor DNA isolated because no biopsy material remained for the purpose. Thus, tumor kinase activity profiles based on previous array phosphosubstrate data were successfully identified for 63 patients that had their tumor KRAS/BRAF/PIK3CA/ERBB2 mutation status determined, and this study population was present within the current analyses.

Tumor Gene Mutation Analyses

KRAS, BRAF, and PIK3CA target sequences were amplified by polymerase chain reaction, and base substitutions were detected by denaturant, cycling temperature capillary electrophoresis [22,23], according to Table S1. ERBB2 amplification was analyzed using the TaqMan® Copy Number Assay (Applied Biosystems, Oslo, Norway) protocol [24] and calibrated relative to each individual patient’s corresponding DNA isolated from peripheral blood mononuclear cells. Tumor DNA samples with relative quantification values higher than 5 were considered amplified to ensure scoring high-grade focal ERBB2 amplification only, omitting low-grade polysomy of chromosome 17.

Tumor Kinase Activity Profiling

Preparation of tumor sample lysates and multiplex analysis of tumor kinase activity using peptide arrays with tyrosine kinase substrates (Tyrosine Kinase PamChip96 Array; PamGene International B.V.). The tumors were divided into two groups; wild-type and mutated KRAS/BRAF (P > 0.67; two-sample t-test). Four technical replicates were analyzed from each patient sample to generate ex vivo phosphosubstrate profiles.

Adaptation of Array Data

Data visualization and processing of previously achieved array data (ArrayExpress accession number E-TABM-913), as reported previously [7], were performed using BioNavigator version 5.10.70 (PamGene International B.V.). The tumors were divided into two groups; wild-type and mutated KRAS/BRAF status (36 and 27 samples, respectively). The data on array peptide phosphorylation,
following conversion from array signal intensities, was log-transformed after handling a small number of negative data points by subtracting the 1% percentile of the total data set and subsequently setting all remaining data points with value less than 1 to the value 1. This adaptation approach was chosen to balance the number of data points that was set to the value of 1 and the extent of collective upward shift of the whole data set. Correction of plate-to-plate variation was achieved by normalizing substrate signal intensity to the mean signal intensity of all wild-type tumors in the respective plates by the following formula: \( N_{\text{psm}} = \log_2(S_{\text{psm}}) - \log_2(G_{\text{psm}}) \), where \( N_{\text{psm}} \) is the normalized signal for substrate \( p \) of sample \( s \) on plate \( m \), \( S_{\text{psm}} \) is the corresponding non-normalized signal, and \( G_{\text{psm}} \) is the average signal from wild-type tumors of substrate \( p \) on plate. Phospho-substrates with a sample-average signal less than 10 were excluded, leaving 102 peptides for further analysis (Table S2).

**Statistical Analysis**

Based on the signal values of these resultant 102 array phosphopeptides, unsupervised analysis was performed applying principal component analysis and \( k \)-means clustering, with 10 Monte Carlo repetitions, using standard functions provided in the Matlab Statistics Toolbox (Matlab R2010A; Mathworks, Natick, MA, USA). Binary supervised classification analysis was performed using partial-least squares discriminant analysis in Matlab R2010A, essentially as described previously [7]. Performance of class partition with respect to tumor mutation status was evaluated by 20-fold cross validation. Distribution of parameters between patients with tumors harboring differential molecular features was compared using Fisher’s exact test for categorical data and two-sample \( t \)-test for continuous variables. Log-rank test was applied to calculate any difference in metastasis-free survival between patient subgroups. The data analysis was performed using SPSS Predictive Analytics Software (SPSS Inc., Chicago, IL, USA). \( P \)-values less than 0.05 were considered statistically significant.

**Results**

**Tumor Mutations**

Point mutations in \( \text{KRAS} \), \( \text{BRAF} \), and \( \text{PIK3CA} \) and amplification of \( \text{ERBB2} \) were detected in 35%, 6.3%, 9.5%, and 3.2% of the primary tumors from 63 LARC cases, respectively (Table 1); in the majority, a single gene aberration was found. Four tumors harbored \( \text{BRAF} \) mutation, either p.D594G or p.V600E, as a solitary aberration. In six tumors, \( \text{PIK3CA} \) mutations were found; in two of the cases, \( \text{KRAS} \) was also mutated. Only two samples, both without other detected mutations, showed amplified \( \text{ERBB2} \) (by virtue of higher than 5-fold tumor \( \text{ERBB2} \) level relative to the level in the patient’s corresponding normal DNA). No differences were observed between patients harboring \( \text{KRAS/BRAF} \) wild-type and mutated tumors regarding radiologic TNM stage at diagnosis, histopathologic ypTN stage or histomorphologic TRG score of the surgical specimens following CRT, development of metastatic disease at median follow-up of 53 months (range 7–70), or age (Table S3).

**Tumor Kinase Activity Profiles**

Ex vivo tumor kinase activity profiles were derived from 102 array substrates that had signal intensities above the defined threshold (Table S2), with relative phosphorylation levels varying within a log2 range of –1.0 to 1.0. Based on the generated phospho-substrate profiles, a binary class partition model discriminated correctly between tumor \( \text{KRAS/BRAF} \) wild-type and mutation status in 67% of cases. No improvement in precision of class partition was achieved on inclusion of either \( \text{PIK3CA} \) or \( \text{ERBB2} \) aberrations as additional layers of information to the group of tumor samples with gene mutations.

Figure 1 shows the score plot resulting from principal component analysis of the data set of 102 phosphopeptide substrates; each spot represents one of the 63 samples in a three-dimensional principal component space. On inspection of the score plot, a single tumor (closed triangle) was observed as a clear outlier to the distribution of samples along the first principal component, and furthermore, the remaining samples seemed to separate into two groups, both consisting of a relatively balanced number of \( \text{KRAS/BRAF} \) wild-type and mutated tumors. Subsequently, using the scores of the three principal components as input and excluding the outlier tumor from further analysis, \( k \)-means clustering was applied in order to obtain two distinct groups of samples, thereby assigning any borderline cases into either of the two. Resulting from this procedure, 15 of 62 samples (24%), of which 11 (69% of the 15) were \( \text{KRAS/BRAF} \) wild-type cases, clustered in the smaller group (Cluster-Group 2; closed circles). Although the larger cluster of 47 samples (Cluster-Group 1; open circles) consisted of 26 (55%) \( \text{KRAS/BRAF} \) wild-type tumors, \( \text{KRAS/BRAF} \) wild-type and mutated cases were equally distributed within the two tumor clusters (\( P = 0.17 \)).

In Figure 2, using the resulting groups from the unsupervised clustering analysis, tumor samples (horizontal axis) and peptides (vertical axis) were sorted along a line connecting the two cluster centroids according to their projection and weight in signal change, respectively, illustrating that Cluster-Group 2 tumors generated higher ex vivo substrate phosphorylation levels than the samples of Cluster-Group 1 for all of the 102 peptides. The order of the peptide substrates with respect to how their difference in phosphorylation levels across the tumor samples distinguished between the two cluster groups, i.e., the discriminating tumor kinase activity profile, is listed in Table 2. Interestingly, differences in phosphorylation of substrates related to PI3K-dependent factors (one \( \text{PIK3R1} \), three \( \text{CTTN1} \), one \( \text{PLCG1} \), two \( \text{PDPK1} \), and one \( \text{RASA1} \) peptide) contributed more strongly to the discrimination

**Table 1. Frequencies of tumor \( \text{KRAS} \), \( \text{BRAF} \), and \( \text{PIK3CA} \) point mutations and \( \text{ERBB2} \) amplification in 63 patients with locally advanced rectal cancer.**

| Mutations       | \( n \) (%) |
|-----------------|-------------|
| **KRAS exon 2** |             |
| p.G12D          | 8 (13)      |
| p.G12V          | 6 (9.5)     |
| p.G13D          | 3 (4.8)     |
| p.G12C          | 2 (3.2)     |
| p.G12S          | 1 (1.6)     |
| p.G13S          | 1 (1.6)     |
| unspecified     | 1 (1.6)     |
| **BRAF exon 15**|             |
| p.D594G         | 2 (3.2)     |
| p.V600E         | 2 (3.2)     |
| **PIK3CA**      |             |
| exon 9          | 5 (7.9)     |
| exon 20         | 2 (3.2)     |
| **ERBB2**       |             |
|                 | 2 (3.2)     |

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of cluster groups, as they essentially were found in the upper half of
the list, than phosphosubstrates related to signaling mediated by
the \textit{KRAS}/\textit{BRAF}-encoded effector pathway (one \textit{RAF1} and four
\textit{MAPK} isoforms peptides). One of the three \textit{EGFR} array peptides
was found among phosphosubstrates strongly distinguishing
between the two cluster groups.

Table 3 summarizes tumor and treatment characteristics of the
62 patients included in either Cluster-Group 1 or Cluster-Group
2. Again, no differences were observed between the cluster groups
regarding TNM stage, ypTN stage, or TRG score. Metastasis-free
survival was assessed for 58 patients, as the four patients with
synchronous liver metastases were omitted from this analysis, with
Cluster-Group 2 demonstrating poorer metastasis-free survival
than Cluster-Group 1 (\(P = 0.011\); Figure 3). Of particular note,
whilst a fifth of patients in Cluster-Group 1 developed metastatic
disease over a follow-up period of 36 months, patients in Cluster-
Group 2 seemed to have a much more aggressive disease, as
almost a half had been diagnosed with metastases by less than one
year of follow-up.

Separately, when comparing \textit{KRAS}/\textit{BRAF} wild-type tumors as a
whole group with the entire group of tumors harboring such
mutations, samples without mutations generated significantly
higher phosphorylation of 11 of the 102 array peptides constituting
the tyrosine kinase activity profiles (\(P\)-value range 0.0034–0.049).

In Figure 4, the samples within each of these two tumor groups are
organized horizontally in order from low to high phosphopeptide
levels, to visualize the higher percentage of \textit{KRAS}/\textit{BRAF} wild-type
tumors that produced higher than mean phosphorylation levels for
the 11 substrates (18 of 36 samples) than tumors with mutated
\textit{KRAS}/\textit{BRAF} performing correspondingly (6 of 27 samples;
\(P = 0.036\)). A majority of the discriminating phosphosubstrates
was deemed to represent signaling factors that are interconnected
with the EGFR-conducted pathway. Within the entire 102-peptide
panel, six peptides representing members of the EGFR family of
receptor tyrosine kinases (three \textit{EGFR}, two \textit{ERBB2}, and one
\textit{ERBB4}) were identified (Table S2). Whilst none of the three
EGFR array peptides was found among phosphosubstrates
distinguishing tumor \textit{KRAS}/\textit{BRAF} mutation status at group level,
all of the three peptides representing \textit{ERBB2} and \textit{ERBB4} were
among the discriminating substrates.

Discussion

The essential finding of this study was that high \textit{ex vivo}
phosphorylation of PI3K-related substrates by the primary tumor,
rather than the \textit{KRAS}/\textit{BRAF} mutation status, may be a hallmark of
poor metastasis-free survival in LARC patients after radical
treatment of the pelvic cavity. This particular subgroup of study

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**Figure 1. Tumor kinase activity cluster groups in patients with locally advanced rectal cancer.** Unsupervised clustering analysis of kinase substrate phosphorylation levels generated by tumors from 63 patients. Distribution of the individual samples of \textit{KRAS}/\textit{BRAF} wild-type (red) and mutated (blue) tumors is visualized using the scores of the first three components in a principal component analysis (PC1–3) of the range of phosphorylation levels of 102 \textit{ex vivo} kinase substrates. \(k\)-means clustering was used to obtain two groups of tumor samples, indicated by open squares (Cluster-Group 1) and closed circles (Cluster-Group 2), respectively. The closed triangle represents a single outlier to the distribution of samples along PC1, as elaborated in Results.
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patients had aggressive disease development, with a substantial fraction of patients being diagnosed with metastatic disease within less than one year of follow-up.

Whilst contemporary multimodal treatment of LARC has led to significant improvement of local disease control, development of metastatic disease is still a major challenge [3]. Currently, no consensus exists to whether systemic therapy may reduce the risk of metastasis development in rectal cancer patients [25], which partly might be explained by the paucity of biomarkers for risk assessment and treatment stratification. In the patient cohort analyzed here, we have previously shown that the presence of disseminated tumor cells in bone marrow at the time of diagnosis correlated with development of overt metastatic disease [8]. The question of whether tumor KRAS/BRAF mutation status may be a reliable biomarker for the purpose of selecting high-risk patients to anti-EGFR therapy, however, remains elusive. Despite convincing evidence of efficacy in metastatic disease from wild-type KRAS/BRAF colorectal tumors [9], including the finding of a high percentage of resectability of liver metastases following cetuximab-based systemic therapy [26], the addition of cetuximab to standard chemotherapy in patients with wild-type KRAS colon cancer failed to meet the endpoint of prolonged disease-free survival in a recently concluded randomized trial in the adjuvant setting [27]. A similar study for resected rectal cancer has not been done.

In the present study, binary supervised classification analysis of the ex vivo-generated phosphopeptide profiles discriminated correctly between tumor KRAS/BRAF wild-type and mutated samples in two-thirds of cases. Because this particular data handling was performed to enable the detection of subtle differences between the two groups being compared, it did not fully compare with the outcome of the unsupervised analysis of the entire 102-phospho-substrate panel. In the latter, two alternative phenotypic tumor populations appeared; a smaller one, comprising a fourth of the entire cohort, and a larger cluster of tumors, both consisting of samples with and without KRAS/BRAF mutations with similar distribution within the two tumor clusters. In metastatic colorectal cancer, objective response to anti-EGFR antibody therapy can be expected in a third of unselected patients, and conversely, tumor KRAS mutations may be found in almost a third of responders [19]. Our observation that KRAS/BRAF wild-type and mutated tumors had overlapping kinase activity profiles is consistent with the increasing recognition of tumor heterogeneity, reflected in disparate mutation status, as determinant of variable response to anti-EGFR antibody therapy.

Figure 2. Tumor ex vivo phosphorylation profiles from patients with locally advanced rectal cancer. An imaginary line was drawn between the determined centroid of each of the patient Cluster-Group 1 and Cluster-Group 2 (depicted in Figure 1), and the 63 tumor samples (horizontal axis; marked for gene mutations as specified) and 102 phosphosubstrates (vertical axis) were sorted along this line according to projection and weight in signal difference, respectively. Red corresponds to higher and blue to lower substrate phosphorylation levels. Arrows denote array peptides representing factors of EGFR-directed signaling pathways, as indicated, and the identity of each peptide substrate, in order from top to bottom of the figure, is given in Table 2. In this analysis, the single outlier to the distribution of samples, as elaborated in Results, sorted left of Cluster-Group 1 in the heat-map.

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Table 2. Order of the 102 array phosphosubstrates, listed from highest to lowest difference in phosphorylation level (top to bottom in Figure 2) between Cluster-Group 1 and Cluster-Group 2 tumors from 62 patients with locally advanced rectal cancer.

| Substrate identity a | Peptide sequence     | Tyrosine position b | Common name a                  |
|----------------------|----------------------|---------------------|---------------------------------|
| PIK3R1               | NENTEDQYSLVED        | [607]               | Phosphatidylinositol 3-kinase regulatory alpha subunit |
| FES                  | REAADGYAASSG         | [713]               | Proto-oncogene tyrosine-protein kinase Fes/Fps |
| CTTN1                | EYEPETYEVAGA         | [477, 483]          | Src substrate protein p85      |
| CDK2                 | EKIGEGTVGYYKH        | [15,19]             | Cell division protein kinase 2 |
| PXN                  | VGEEDYVYSPFPNK       | [118]               | Paxillin                       |
| VEGFR2 (KDR)         | EEAPEDLKDFL          | [996]               | Vascular endothelial growth factor receptor 2 |
| EPHA2                | EDDPEATYTTSGG        | [772]               | Ephrin type-A receptor 2       |
| EPHA1                | LDDFDGYETQGG         | [781]               | Ephrin type-A receptor 1       |
| PXN                  | FLSEETYPSPTG         | [31,33]             | Paxillin                       |
| PECAM1               | KKDTELTVSEVRK        | [713]               | Platelet endothelial cell adhesion molecule |
| EPHA7                | TVIDPETYEDP            | [608, 614]          | Ephrin type-A receptor 7       |
| CD247                | KDKMAEAYSEGM         | [123]               | T-cell surface glycoprotein CD3 zeta chain |
| FRK                  | KVNDIEDYESRHE        | [387]               | Tyrosine-protein kinase FRK    |
| EPHB1                | DDDSDPTTTSSLG        | [778]               | Ephrin type-B receptor 1       |
| EPOR                 | ASAASEYTLDP          | [426]               | Erythropoietin receptor        |
| RET                  | TPSDLDYGDSL          | [1029]              | Proto-oncogene tyrosine-protein kinase receptor ret |
| EPOR                 | SEHAQTDYVLKD         | [368]               | Erythropoietin receptor        |
| PDGFRB               | VSSDGHEYVYDP         | [579, 581]          | Beta platelet-derived growth factor receptor |
| LAT                  | EEEAPDNENQEL         | [255]               | Linker for activation of T cells |
| FER                  | RQEDGGYSSSGL         | [714]               | Proto-oncogene tyrosine-protein kinase FER |
| EGFR                 | GSVQNPVYHQLQ         | [1110]              | Epidermal growth factor receptor |
| PLCG1                | IGTAEPDYGALYE        | [771, 775]          | 1-phosphatidylinositol-4,5-bisphosphate phosphodiesterase gamma 1 |
| PDK1                 | ARTTQLYDAVPI         | [9]                 | 3-phosphoinositide dependent protein kinase 1 |
| PDGFRB               | PNEGONDYIPLDP        | [1021]              | Beta platelet-derived growth factor receptor |
| CBL                  | EGEEDEYMTSSS         | [700]               | CBL E3 ubiquitin protein ligase |
| LAT                  | MESIDDYVNPES         | [200]               | Linker for activation of T cells |
| PDGFRB               | SSSNYMAPDNYVP         | [771, 775, 778]     | Beta platelet-derived growth factor receptor |
| PDGFRB               | LDTSSVLTYAVQ         | [1009]              | Beta platelet-derived growth factor receptor |
| TNNT1                | SDTEEEQYEEQDP        | [9]                 | Slow skeletal muscle tropinonT |
| KRIT6E               | GACFGSRVYSLG         | [62]                | Keratin, type II cytoskeletal 6E |
| RASA1                | TVDGKEYINTIR         | [460]               | Ras GTPase-activating protein 1 |
| PDGFRB               | YMAPDNYVPSAP         | [771, 775, 778]     | Beta platelet-derived growth factor receptor |
| ANXA2                | HSTPPSASYGSKA        | [24]                | Annexin A2                     |
| PTK2B                | RYIEEDDYKAVS         | [573, 579, 580]     | Protein tyrosine kinase 2 beta |
| PDGFRB               | RPPSALSYNSALP        | [716]               | Beta platelet-derived growth factor receptor |
| JAK1                 | AIETDKEYTVKD         | [1022, 1023]        | Tyrosine-protein kinase JAK1   |
| ZAP70                | ALGADDSYTTARS        | [492, 493]          | Tyrosine-protein kinase ZAP-70 |
| DDR1                 | LLLSNPARYLLLA        | [513]               | Epithelial discoidin domain receptor 1 |
| CTNNB1               | VADIIDQYAMTRA        | [86]                | Beta-catenin                    |
| JAK2                 | VRREVGYQGQLHETE      | [570]               | Tyrosine-protein kinase JAK2   |
| CTTN1                | YQAENETYDEVEN        | [492, 499, 502]     | Src substrate protein p85      |
| FGRF2                | TLTTNELYLDLSQ        | [769]               | Fibroblast growth factor receptor 2 |
| MET                  | RDMYDKEYYSVHN        | [1230, 1234, 1235]  | Hepatocyte growth factor receptor |
| ART-004              | EAYAAPFAKKK          | [4]                 | Artificial peptide sequence    |
| NTRK2                | GMSRDVYSTDYR         | [702, 706, 707]     | BDNF/NT-3 growth factor receptors |
| VEGFR1 (FLT1)        | DYSVWLYSTPPI         | [1327, 1333]        | Vascular endothelial growth factor receptor 1 |
| Substrate identity * | Peptide sequence | Tyrosine position b | Common name a |
|----------------------|------------------|--------------------|---------------|
| ANXA1                | IENEEQEYQTVK     | [21]               | Annexin A1    |
| M5T1R                | SALLGDHYQVLPA    | [1353]             | Macrophage-stimulating protein receptor |
| LCK                  | RLI6DEY7AEG      | [394]              | Proto-oncogene tyrosine-protein kinase LCK |
| VEGFR2 (KDR)         | AQQDGKDYIVLPI    | [1175]             | Vascular endothelial growth factor receptor 2 |
| ERBB2                | LDIDETEYHADGG    | [877]              | Receptor tyrosine-protein kinase erbB-2 |
| MAPK7                | AEHQYFMTYEYAT    | [215, 220]         | Mitogen-activated protein kinase 7 |
| PDPK1                | DEDCYGN1DNLS     | [373, 376]         | 3-phosphoinositide dependent protein kinase 1 |
| PRRX2                | W7ASSYPX5VPPY    | [208, 214]         | Paired mesoderm homeobox protein 2 |
| EGLR                 | ISLDNPXYQDDOF    | [1172]             | Epidermal growth factor receptor |
| CTNN1                | VSQREAYEPEVT     | [477]              | Src substrate protein p85 |
| M5T1R                | YVQLPATYMNGLP    | [1353, 1360]       | Macrophage-stimulating protein receptor |
| EPB41                | LDGENIY1HRHSNL   | [660]              | Protein 4.1 |
| CHRN1                | Y1SKAEYFLKIS     | [383, 390]         | Acetylcholine receptor protein, delta subunit |
| ERBB2                | PTX16NPXGLDV     | [1248]             | Receptor tyrosine-protein kinase erbB-2 |
| EGLR                 | STAENAEYLRVAP    | [1197]             | Epidermal growth factor receptor |
| CALM1                | K1D1NGY1SAARL    | [100]              | Calmodulin |
| FGFR1                | TSNQY1IDL1SMPL   | [766]              | Basic fibroblast growth factor receptor 1 |
| DCX                  | GIVY1VSSD1FRS    | [112]              | Neuronal migration protein doublecortin |
| FGFR3                | TV1STDIY1DL1SA   | [760]              | Fibroblast growth factor receptor 3 |
| VEGFR1 (FLT1)        | ATSMFDPY1QDDSS   | [1242]             | Vascular endothelial growth factor receptor 1 |
| TEC                  | RYFLDQY1TSSSG    | [513, 519]         | Tyrosine-protein kinase Tec |
| RAF1                 | PRQR0DSSYYWSI    | [340, 341]         | RAF proto-oncogene serine/threonine-protein kinase |
| PGR                  | EQRK1SSFY1SLC    | [795]              | Progesterone receptor (PR) |
| BK10HA               | DDSSAYSVD1EVN    | [345]              | 2-oxoisovalerate dehydrogenase alpha subunit, mitochondrial |
| MAPK10               | TSFM1MT1PPYV1T1Y | [223]              | Mitogen-activated protein kinase 10 |
| D1YR1K1A             | C1QG1RIY1Q1YQS   | [319, 321]         | Dual-specificity tyrosine-phosphorylation regulated kinase 1A |
| ERBB4                | IVAENPEY1LSEFS   | [1284]             | Receptor tyrosine-protein kinase erbB-4 |
| VEGFR2 (KDR)         | DIY1KDPY1VR1GKD  | [1054, 1059]       | Vascular endothelial growth factor receptor 2 |
| RB1                  | I1Y1SPLK1SS1KIS  | [805, 813]         | Retinoblastoma-associated protein |
| MAPK1                | HTGFL1TEY1VTRW   | [187]              | Mitogen-activated protein kinase 1 |
| INSR                 | Y1ASSNPY1L1SAD  | [992, 999]         | Insulin receptor |
| PTK2                 | RYMED1ST1YY1KASK | [570, 576, 577]    | Focal adhesion kinase 1 |
| EPHA4                | LN1QG1R11TV1DPF1 | [596]              | Ephrin type-A receptor 4 |
| EPHA8                | I1GH1TKY11DPF1   | [590]              | Ephrin type-B receptor 4 |
| VCL                  | K15FLD1G1RY1LGA  | [822]              | Vinculin |
| SYK                  | AL1RAD1EN1Y1QAOT | [525, 526]         | Spleen tyrosine kinase |
| VEGFR1 (FLT1)        | DF1GL1ARD1Y1KN1PD| [1048]             | Vascular endothelial growth factor receptor 1 |
| C1R                  | TEASGY1ISS1L1YP  | [204, 210]         | Complement C1r subcomponent |
| MBP                  | ARTAH1YG1SLPQKS  | [203]              | Myelin basic protein |
| PPP2CB               | E1HP1HR11TP1DYF1 | [307]              | Serine/threonine protein phosphatase 2A, catalytic subunit, beta isoform |
| VEGFR2 (KDR)         | DF1GL1ARD1Y1KDPD| [1063]             | Vascular endothelial growth factor receptor 2 |
| MAPK1                | AD1SEM1TGY1V1TRW | [185]              | Mitogen-activated protein kinase 12 |
| SLC34A1              | AK1ALGKR1AT1K1RW | [511]              | Renal sodium-dependent phosphate transport protein 2 |
| Z1BT16               | L1RTH1NG1AS1PYQCT| [630]              | Zinc finger and BTB domain containing protein 16 |
Of notice, study patients demonstrating high tumor PI3K-mediated signaling activity, as all array substrates of this specific pathway (PIK3R1, CTTN1, PLCG1, PDPK1, and RASA1) were highly phosphorylated, had particularly poor metastasis-free survival after radical treatment of the pelvic cavity. The PI3K complex consists of a regulatory subunit, existing in several isoforms (PIK3R1 and CTTN1), and a catalytic subunit encoded by \textit{PIK3CA}. On regulatory subunit phosphorylation by receptor

| Substrate identity | Peptide sequence | Tyrosine position | Common name |
|--------------------|------------------|-------------------|-------------|
| CDK7               | GLAKSFGSNPARY    | [169]             | Cell division protein kinase 7 |
| VEGFR3 (FLT4)      | DIYKDPDVDVRGKS   | [1063, 1068]      | Vascular endothelial growth factor receptor 3 |
| TYRO3              | KYSGDYYRQGCA     | [681, 685, 686]   | Tyrosine-protein kinase receptor TYRO3 |
| VEGFR2 (KDR)       | RFRQGKDYYGAIP    | [951]             | Vascular endothelial growth factor receptor 2 |
| NCF1               | QRSKRRLSDQDAYR   | [324]             | Neutrophil cytosol factor 1 |
| MBP                | FCYGGGRASDYKA    | [261, 268]        | Myelin basic protein |
| PTPN11             | SKRKGHEYTNIKY    | [546, 551]        | Tyrosine-protein phosphatase, non-receptor type 11 |
| NTRK1              | HIEIPOYFSDAC     | [496]             | High affinity nerve growth factor receptor |
| MBP                | GRASDYSAHKGF     | [268]             | Myelin basic protein |
| ENPEP              | EREGSKRCIQTK     | [12]              | Glutamyl aminopeptidase |
| VEGFR1 (FLT1)      | KNPDYVRGKDTFL    | [1053]            | Vascular endothelial growth factor receptor 1 |
| INSR               | SLGFKRSYEHHIP    | [1355]            | Insulin receptor |

Peptides representing phosphatidylinositol-3-kinase-dependent factors are indicated in bold, whereas peptides related to signaling mediated by the KRAS/BRAF-encoded effector pathway are italicized. The EGFR peptides are highlighted in bold and italics.

\*Retrieved from UniProtKB/SwissProt (http://au.expasy.org/sprot).
\#Position(s) of the tyrosine phosphorylation site(s) within the protein.

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Figure 3. Metastasis-free survival in locally advanced rectal cancer. This outcome parameter was analyzed for 58 patients as function of low (Cluster-Group 1) or high (Cluster-Group 2) ex vivo substrate phosphorylation activity of the primary tumor.
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tyrosine kinases or G-protein (RASA1)-coupled receptors, the catalytic subunit is enabled to generate phosphatidylinositol-3,4,5-trisphosphate, which activates 3-phosphoinositide-dependent protein kinase 1 (PDPK1) and subsequently AKT and the downstream mammalian target of rapamycin (mTOR). In addition, the 1-phosphatidylinositol-4,5-bisphosphate phosphodiesterases

**Table 3. Tumor and treatment characteristics of 62 patients with locally advanced rectal cancer.**

|               | All patients in sample clusters (n=62) | Patients in Cluster Group 1 (n=47) | Patients in Cluster Group 2 (n=15) |
|---------------|---------------------------------------|-----------------------------------|-----------------------------------|
| **TNM**       |                                       |                                   |                                   |
| T2            | 4 (6.5%)                              | 4 (8.5%)                          | 0 (0%)                            |
| T3            | 36 (58%)                              | 29 (62%)                          | 7 (47%)                           |
| T4            | 22 (35%)                              | 14 (30%)                          | 8 (53%)                           |
| N0            | 8 (13%)                               | 8 (17%)                           | 0 (0%)                            |
| N1            | 9 (15%)                               | 6 (13%)                           | 3 (20%)                           |
| N2            | 45 (73%)                              | 33 (70%)                          | 12 (80%)                          |
| M0            | 57 (92%)                              | 44 (94%)                          | 13 (87%)                          |
| M1            | 5 (8.1%)                              | 3 (6.4%)                          | 2 (13%)                           |
| **ypTN**      |                                       |                                   |                                   |
| ypT0          | 13 (21%)                              | 10 (21%)                          | 3 (20%)                           |
| ypT1          | 6 (10%)                               | 3 (6.4%)                          | 3 (20%)                           |
| ypT2          | 16 (26%)                              | 15 (32%)                          | 1 (6.2%)                          |
| ypT3          | 15 (24%)                              | 12 (26%)                          | 3 (20%)                           |
| ypT4          | 12 (19%)                              | 7 (15%)                           | 5 (33%)                           |
| ypN0          | 49 (79%)                              | 38 (81%)                          | 11 (73%)                          |
| ypN1          | 10 (16%)                              | 8 (17%)                           | 2 (13%)                           |
| ypN2          | 3 (4.8%)                              | 1 (2.1%)                          | 2 (13%)                           |
| **TRG** a     |                                       |                                   |                                   |
| 1–2           | 45 (73%)                              | 35 (74%)                          | 10 (67%)                          |
| 3             | 9 (15%)                               | 8 (17%)                           | 1 (6.7%)                          |
| 4–5           | 8 (13%)                               | 4 (8.5%)                          | 4 (27%)                           |
| Development of metastatic disease b | 16 (26%) | 9 (20%) | 7 (50%) |

*aTumor Regression Grade following chemoradiotherapy.

*bCensored at a median period of 53 months (range 7–70), excluding four patients with synchronous resectable liver metastases.

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**Figure 4. Tumor ex vivo phosphorylation profiles discriminating tumor KRAS/BRAF mutation status in locally advanced rectal cancer.**

The 63 tumor samples are ordered along the horizontal axis, annotated by wild-type or mutated KRAS/BRAF and marked for other gene mutations as specified, while the 11 discriminating kinase substrates (P-value range 0.0034–0.049 on comparison of KRAS/BRAF wild-type tumors as a whole group with the entire group of tumors harboring such mutations) are depicted along the vertical axis. For each peptide substrate, position(s) of the tyrosine phosphorylation site(s) within the protein is indicated. Red corresponds to higher and blue to lower substrate phosphorylation levels.

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esterase gamma-1 (PLCG1) is crucial for generation of activating second messenger molecules in the EGFR-directed, PIK3-mediated signaling pathway [17,18]. In the context of our observations, even without valid evidence at present, it is tempting to speculate that LARC patients might be eligible for adjuvant systemic therapy based on this high-risk biological feature. Given the finding that the PI3K complex may be a key signaling network orchestrator of colorectal cancer metastasis, therapeutics targeting PI3K/AKT or the downstream mTOR complex [17] might be rational, and within this frame of reference, the ex vivo phosphosubstrate technology could show useful in developing the required biomarkers of signaling pathway druggability.

In the separate analysis comparing ex vivo phosphopeptide profiles generated by KRAS/BRAF wild-type tumors as a whole group with those collectively obtained from the group of tumors harboring such mutations, ERBB2 and ERBB4 were among the prevailing substrates discriminating these two groups. However, bearing in mind that the 11 discriminating substrates in this analysis appeared from a total number of 102 peptides constituting the tyrosine kinase activity profiles, the false discovery rate might be as high as 50% with the statistical significance level of P<0.05.

Nevertheless, resistance to anti-EGFR antibody treatment may be mediated by activation of ERBB2-mediated signaling, either via amplification of ERBB2 or increased levels of the ERBB3/ERBB4 ligand heregulin [13]. Moreover, ERBB2 was recently found to be amplified in a third of tumors, predominantly colon cancer, confirmed to be wild-type for KRAS/BRAF/PIK3CA but resistant to anti-EGFR antibody therapy; tumors with ERBB2 amplification were substantially enriched in this specific population compared to unselected patients [12]. In the present cohort of LARC patients, however, only two cases were concluded to have ERBB2 amplification.

Specifically, using high-throughput kinase substrate arrays, an association between tumor kinase activity and metastasis-free survival was found in this LARC cohort. For clinical practice, this technology may be practicable, as it is robust with small tissue quantities, typically 10–15 micrograms of total protein being sufficient [4–6]; however, it has so far been employed to address a limited number of clinical topics [7,8,28–30]. The concept is contingent on fresh-frozen tumor tissue for preservation of kinase activity, and for the investigation reported here, we took the advantage of an existing biobank of biopsy samples prospectively compiled from study patients, enabling analysis of quality-assured tumor tissue. However, the present LARC population had not received anti-EGFR antibody treatment and thus, such outcome data was unavailable for correlation to the generated tumor kinase activity profiles.

In selecting cancer patients to kinase inhibiting therapeutics, the prevailing gold-standard is based mainly on detection of gene aberrations in the patients’ tumors. Such defects are embodied as activating or inhibiting, or as amplifications or translocations, and are currently utilized in colorectal, breast, and non-small cell lung carcinomas, malignant melanoma, gastrointestinal stromal tumor, and some hematologic malignancies [19]. In the LARC population studied here, the observed frequencies of tumor aberrations of KRAS, BRAF, PIK3CA, and ERBB2 were in the order of magnitude previously reported in colorectal cancer [9,12,17,31]. Also in accordance with previous observations [9], mutations in KRAS and BRAF were mutually exclusive, whereas KRAS and PIK3CA mutations could coexist. The tumor from one patient harbored no less than four detected mutations (KRAS p.G12S, one PIK3CA exon 9 mutation, and two PIK3CA exon 20 mutations). Moreover, the lack of correlation between tumor KRAS/BRAF mutation status and treatment outcome for the present study population is in agreement with previous reports of other patient cohorts treated with neoadjuvant fluoropyrimidine-/oxaliplatin-based CRT [32–34]. Whether tumor KRAS mutation status is predictive for cetuximab-based CRT in LARC, is presently under debate [35–38].

In conclusion, recognizing that high tumor PI3K-mediated signaling activity was associated with poor metastasis-free survival in LARC, the strategy of exploring tumor kinase activities might be used to define functional biomarkers for risk assessment and treatment stratification. The present analysis needs to be repeated in more comprehensive patient populations, preferably with validated outcome data from adjuvant therapy, to ultimately prove diagnostic value for identification of patients with highly aggressive disease. Alternatively, as research tool, this approach for analyzing composite activities of signaling pathway effector proteins may be further developed to study actionable targets for prevention or treatment of colorectal cancer metastasis in general.

Supporting Information

Table S1 Specifications of the tumor KRAS, BRAF, and PIK3CA mutation analyses.

Table S2 The 102 array substrates generating the ex vivo tumor kinase activity signatures.

Table S3 Characteristics of 63 study patients with locally advanced rectal cancer with regard to tumor KRAS/BRAF mutation status.

Author Contributions

Conceived and designed the experiments: AHR ATK RdW HE KF.Performed the experiments: AHR ATK MGS RdW JJ TWA. Analyzed the data: AHR MGS RdW HE SD KF. Contributed reagents/materials/analysis tools: AHR ATK RdW HE TWA SD KF. Wrote the paper: AHR. Management of patients, databases, and tissue banking: AHR MGS TWA SD KF. Development and validation of technical procedures: JJ TWA.
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