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Original contribution

EGFR gene copy number decreases during anti-EGFR antibody therapy in colorectal cancer☆,☆☆

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Summary Epidermal growth factor receptor (*EGFR*) gene copy number (GCN) increase is associated with a favorable anti-EGFR antibody treatment response in *RAS* wild-type metastatic colorectal cancer. However, there are limited and comparative data regarding the *EGFR* GCN in primary colorectal cancer tumors and corresponding metastases or the effect of anti-EGFR antibody treatment on *EGFR* GCN in recurrent disease. In addition, little is known about the potential *EGFR* GCN changes during anti-EGFR therapy in comparison with other treatment regimens. *EGFR* GCN was analyzed by EGFR immunohistochemistry-guided silver in situ hybridization in primary and corresponding recurrent local or metastatic tumors from 80 colorectal cancer patients. GCN levels were compared between *KRAS* wild-type patients having received anti-EGFR therapy and patients having received other forms of treatment after primary surgery. The *EGFR* GCN decrease between primary and recurrent tumors was more pronounced among the anti–EGFR-treated patients than among patients not treated with anti-EGFR therapy ($P = .047$). None of the patients experiencing an *EGFR* GCN increase of at least 1.0 between the primary and recurrent tumors were treated with anti-EGFR antibodies. When including only patients with distant metastases, an *EGFR* GCN decrease of at least 1.0 was more common among the anti–EGFR-treated patients than among patients not treated with anti-

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EGFR therapy ($P = .028$). Our results suggest that anti-EGFR antibody treatment is associated with *EGFR* GCN decrease between the primary and recurrent colorectal adenocarcinomas, whereas no GCN change is observed among patients receiving other forms of treatment after primary surgery.

1. Introduction

Epidermal growth factor receptor (EGFR) is a transmembrane receptor tyrosine kinase that can initiate several intracellular signaling pathways contributing to cancer cell proliferation, inhibition of apoptosis, invasion, metastasis, and stimulation of neovascularization. The main regulatory routes of EGFR are the RAS-RAF-MAPK and the PI3K-Akt signaling pathways [1]. The central role of EGFR in cancer progression is used in the treatment of *RAS* wild-type metastatic colorectal cancer (CRC) by EGFR-targeting monoclonal antibodies cetuximab and panitumumab [2-7]. In addition to *RAS* status, an *EGFR* gene copy number (GCN) of at least 4.0 in the primary tumor has been associated with a favorable anti-EGFR antibody treatment response in patients with *RAS* wild-type CRC [8-10]. However, there are limited comparative data regarding the *EGFR* GCN in the primary CRC tumors and the corresponding metastases, and few studies have examined the effect of anti-EGFR antibody treatment on the *EGFR* GCN in the metastatic tumors. Here, we analyzed the concordance of *EGFR* GCN between primary and recurrent CRC tumors in patients treated with anti-EGFR therapy in comparison with patients receiving other forms of treatment after primary surgery.

2. Materials and methods

2.1. Patients and clinical tumor material

The study population in this retrospective study consists of 80 patients treated for CRC at the Turku University Hospital (n = 66) and Central Finland Central Hospital (n = 14) between 2000 and 2015. Three of the Turku patients had their liver metastasis resections performed at the Helsinki University Hospital. The inclusion criteria were diagnosis of adenocarcinoma in the colon or rectum and the availability of both primary and recurrent tumor material for the study purposes. The recurrent tumors were either local recurrences or distant metastases. The median age of the patients at the time of diagnosis was 66 years (range, 34-86 years). Most of the patients (58.8%) had *KRAS* wild-type tumors. The *KRAS* mutation testing was performed in primary tumors for 76 patients and in recurrent tumors for 3 patients. For 1 patient, both the primary and recurrent tumors were tested for clinical purposes.

Altogether 24 of 80 patients were treated with anti-EGFR therapy. Of those, the relationship between *EGFR* GCN change and anti-EGFR antibody treatment was analyzed in *KRAS* wild-type CRC patients whose primary tumor samples were obtained before and recurrent tumor samples after the administration of anti-EGFR therapy (n = 14). The patients received cetuximab or panitumumab either as single therapy or in combination with irinotecan. The *EGFR* GCN of their tumor samples was compared with the samples of patients receiving adjuvant chemotherapy or no adjuvant therapy after primary surgery. One patient was excluded from the analyses because the sample taken before anti-EGFR treatment was obtained from the metastatic site. In addition, 1 anti–EGFR-treated patient was excluded from the analyses regarding antibody therapy because the primary tumor was *KRAS* mutated.

The response to anti-EGFR antibody treatment was evaluated in 13 patients receiving antibodies before the sample was obtained from the recurrent tumor. The evaluation was performed by computed tomography or magnetic resonance imaging according to the Response Evaluation Criteria in Solid Tumors [11]. The median follow-up time of all the patients was 97 months (range, 8-174 months). At the end of the follow-up period, 28% (22/80) of the patients were alive. The reporting of the study has been performed in compliance with the current recommendations [12]. The patient and tumor characteristics are presented in more detail in Table 1.

The study was conducted in accordance with the Declaration of Helsinki and the Finnish legislation for the use of archived tissue specimens and associated clinical information. The clinical data were retrieved, and the histologic samples were collected and analyzed with the endorsement of the National Authority for Medico-Legal Affairs and the institutional review board of the Hospital District of Southwest Finland. Oral or written informed consent was not obtained because most of the patients included in this study had died of their disease. The need for informed consent from participants was waived by the National Authority for Medico-Legal Affairs.

2.2. *KRAS* mutation testing

For all patients, *KRAS* point mutations within codons 12, 13, and 61 were detected from formalin-fixed, paraffin-embedded (FFPE) tissue material containing at least 30% of CRC cells. For 60 (75%) patients, the *KRAS* gene was analyzed with pyrosequencing technique using the IVD marked Qiagen TheraScreen KRAS Pyro kit (catalog no. 971460; Qiagen GmbH, Hilden, Germany). The testing was performed according to the protocol provided by the manufacturer. Shortly, 10-μm-thick FFPE tissue slices (5-10 per sample) were collected to a microsentrifuge tube, and the DNA

Table 1 Clinicopathological characteristics of CRC patients with either local recurrence or distant metastasis

	Only local recurrence, n (%)	Distant metastasis, n (%)[a]	All, n (%)
No. patients	21 (26.3)	59 (73.8)	80 (100.0)
Patient sex			
Female	11 (52.4)	27 (45.8)	38 (47.5)
Male	10 (47.6)	32 (54.2)	42 (52.5)
Age at diagnosis (y)			
Median	63	69	66
Range	34-82	38-87	34-87
KRAS status			
Wild-type	10 (47.6)	37 (62.7)	47 (58.8)
Mutated	11 (52.4)	22 (37.3)	33 (41.3)
Tumor differentiation grade			
Grade 1	2 (9.5)	7 (11.9)	9 (11.3)
Grade 2	15 (71.4)	40 (67.8)	55 (68.8)
Grade 3	4 (19.0)	10 (16.9)	14 (17.5)
No information	0 (0.0)	2 (3.4)	2 (2.5)
Site of primary tumor			
Colon	12 (57.1)	28 (47.5)	40 (50.0)
Rectum	9 (42.9)	31 (52.5)	40 (50.0)
Location of primary tumor			
Left-sided (descendens-rectum)	13 (61.9)	48 (81.4)	61 (76.3)
Right-sided (cecum-transversum)	8 (38.1)	11 (18.6)	19 (23.8)
Stage at diagnosis			
I	2 (9.5)	5 (8.5)	7 (8.8)
II	10 (47.6)	17 (28.8)	27 (33.8)
III	9 (42.9)	33 (55.9)	42 (52.5)
IV	0 (0.0)	4 (6.8)	4 (5.0)
Preoperative (chemo)radiotherapy			
Short-course	0 (0.0)	10 (16.9)	10 (12.5)
Long-course	3 (14.3)	4 (6.8)	7 (8.8)
No therapy	18 (85.7)	45 (76.3)	63 (78.8)
Type of surgery			
Right hemicolectomy	8 (38.1)	8 (13.6)	16 (20.0)
Left hemicolectomy	0 (0.0)	4 (6.8)	4 (5.0)
Anterior resection	10 (47.6)	26 (44.1)	36 (45.0)
APR	3 (14.3)	15 (25.4)	18 (22.5)
Other	0 (0.0)	6 (10.2)	6 (7.5)
Postoperative treatment			
Adjuvant chemotherapy and radiotherapy	2 (9.5)	7 (11.9)	9 (11.3)
Only adjuvant chemotherapy	12 (57.1)	31 (52.5)	43 (53.8)
Only radiotherapy	0 (0.0)	1 (1.7)	1 (1.3)
No treatment	7 (33.3)	20 (33.9)	27 (33.8)
Recurrent disease			
Local recurrence only			21 (26.3)
Distant metastases			
Liver			30 (37.5)
Lung			6 (7.5)
Other distant			13 (16.3)
Local and distant			4 (5.0)
Multiple			6 (7.5)
Type of metastasis			
Synchronous (<6 mo after dg)	0 (0.0)	5 (8.5)	5 (6.3)
Metachronous (≥6 mo after dg)	21 (100.0)	54 (91.5)	75 (93.8)
Anti-EGFR antibody treatment			
Yes	5 (23.8)	19 (32.2)	24 (30.0)
No	16 (76.2)	40 (67.8)	56 (70.0)

(continued on next page)

Table 1 (*continued*)

	Only local recurrence, n (%)	Distant metastasis, n (%)[a]	All, n (%)
Anti-EGFR antibody treatment before recidive resection			
Yes	1 (4.8)	15 (25.4)	16 (20.0)
No	20 (95.2)	44 (74.6)	64 (80.0)
Follow-up status			
Alive	7 (33.3)	15 (25.4)	22 (27.5)
Dead	14 (66.7)	44 (74.6)	58 (72.5)

Abbreviations: APR, abdominoperineal resection; dg, diagnosis.
[a] Including 4 patients with both local and distant recurrent tumors.

was extracted with QIAamp DNA FFPE tissue kit (Qiagen). Next, 2 separate polymerase chain reactions were performed targeting codons 12, 13, and 61. The polymerase chain reaction products were then sequenced using the PyroMark Q24 (Qiagen). Data analysis was performed with the PyroMark Q24 analysis program (Qiagen). For 20 patients (25%), DxS K-RAS Mutation kit (DxS Ltd, Manchester, UK) was used for the analysis.

2.3. EGFR immunohistochemistry and silver in situ hybridization

The EGFR immunohistochemistry (IHC) staining and *EGFR* GCN analysis procedures have been described previously [8-10]. Briefly, EGFR IHC staining was performed on 3-μm sections with a monoclonal antibody against the internal domain of EGFR (Ventana Medical Systems/Roche Diagnostics, Tucson, AZ; clone 5B7). The stainings were executed with BenchMark XT (Ventana/Roche) using *ultra*View Universal DAB Detection Kit (Ventana/Roche). *EGFR* gene was detected from 5-μm sections with *EGFR* DNA Probe (Ventana/Roche), and silver in *situ* hybridization (SISH) was performed with the BenchMark XT using *ultra*VIEW SISH Detection Kit (Ventana/Roche). *EGFR* GCN was calculated as a mean value from 40 tumor cells from the areas of highest IHC reactivity in each tumor.

When calculating the *EGFR* GCN change between the primary and corresponding metastatic tumors, a cutoff value

Table 2 *EGFR* GCN in the primary and corresponding recurrent tumors detected by SISH

	Primary tumors (n = 78)	Recurrent tumors (n = 79)	*P*
EGFR GCN (continuous, all patients)[a]			
Mean (95% CI)	4.42 (3.94-4.89)	4.25 (3.80-4.71)	.268[e]
Median (range)	3.98 (2.00-15.00)	3.80 (2.30-13.5)	
Anti-EGFR treatment (n = 14)[b]			
Mean (95% CI)	5.55 (3.36-7.74)	3.92 (2.52-5.31)	.028[e,*]
Median (range)	4.26 (2.79-15.0)	3.43 (2.36-12.0)	
Without anti-EGFR treatment (n = 63)[c]			
Mean (95% CI)	4.20 (3.83-4.56)	4.33 (3.84-4.82)	.771[e]
Median (range)	3.90 (2.00-10.0)	3.97 (2.30-13.5)	
EGFR GCN (dichotomous, all patients)[d]			
<4.0 (%)	39 (50.0)	43 (55.1)	.572[f]
≥4.0 (%)	39 (50.0)	35 (44.9)	
Anti-EGFR treatment (n = 14)[b]			
<4.0 (%)	5 (35.7)	11 (78.6)	.031[f,*]
≥4.0 (%)	9 (64.3)	3 (21.4)	
Without anti-EGFR treatment (n = 63)[c]			
<4.0 (%)	33 (52.4)	32 (50.8)	>.999[f]
≥4.0 (%)	30 (47.6)	31 (49.2)	

[a] Missing SISH information from 1 primary tumor and excluding 1 tumor pair with pre–anti-EGFR treatment sample obtained from the metastatic site.
[b] Excluding 1 patient with *KRAS*-mutated primary tumor. Administered with or without chemotherapy after primary surgery but before obtaining the sample from the recurrent tumor.
[c] No anti-EGFR antibody treatment before obtaining the sample from the recurrent tumor.
[d] Excluding 1 patient with missing SISH information from the primary tumor and one patient with pre–anti-EGFR treatment sample obtained from the metastatic site.
[e] Wilcoxon signed-rank test.
[f] McNemar test.
* $P < .05$.

of 1.0 was used to signify a notable alteration in the GCN. In addition to the cutoff value of 1.0 for *EGFR* GCN change, we tested the cutoff value 4.0, which we have previously shown to predict anti-EGFR treatment response in metastatic CRC [8]. EGFR IHC and *EGFR* GCN were scored independently by 2 observers (J. S. and T. A., E. B., or M. L.) without knowledge of the clinical information. In cases of discordance, a consensus was made between the 2 observers. *EGFR* GCN could not be analyzed in one primary tumor because the hybridization reaction was too weak.

2.4. Statistical analyses

Statistical analyses were performed with IBM SPSS Statistics for Windows, version 24.0 (IBM Corporation, Armonk, NY). Frequency table data were analyzed with the Pearson χ^2 test or Fisher exact test for categorical variables. Tables of 2 × 2 were used to calculate odds ratios (ORs) and 95% confidence intervals (CIs) using the exact method. To compare the mean GCN in relation to categorical variables, nonparametric Mann-Whitney and Kruskal-Wallis tests were used because the *EGFR* GCN was not normally distributed. Pairwise concordance of *EGFR* GCN between primary and metastatic tumors was analyzed using a nonparametric paired-samples test (McNemar and Wilcoxon signed-rank test). Kaplan-Meier method with log-rank test and Cox proportional hazards regression model were used for univariate survival analysis. Disease-free survival was calculated from the time of diagnosis to the time of first recurrence, death of primary cancer, or the last follow-up date. Overall survival was calculated from the time of diagnosis to the time of death of any cause or the last follow-up date. All statistical tests were 2-sided, and *P* values less than .05 were considered statistically significant.

3. Results

3.1. *EGFR* GCN in the primary and recurrent tumors

Among the primary tumors (n = 78), the median *EGFR* GCN was 3.98, and in the recurrent tumors (n = 79), the median *EGFR* GCN was 3.80. The median change in *EGFR* GCN was −0.14 units during disease progression, which is

Table 3 *EGFR* GCN change between the primary and corresponding recurrent tumors detected by SISH

EGFR GCN change	All patients (n = 78) [a]	Anti-EGFR treatment [b] (n = 14)	Without anti-EGFR treatment [c]			
			Local recidives and distant metastases (n = 63) [a]	*P* [d]	Only distant metastases (n = 44)	*P* [d]
Absolute						
Mean (95% CI)	−0.19 (−0.76 to 0.38)	−1.63 (−3.52 to 0.25)	0.10 (−0.46 to 0.67)	.073	0.26 (−0.44 to 0.95)	.096
Median (range)	−0.14 (−11.7 to 9.40)	−0.46 (−11.7 to 0.70)	−0.10 (−7.70 to 9.40)		−0.14 (−4.55 to 9.40)	
Relative (%)						
Mean (95% CI)	4.09 (−6.21 to 14.4)	−19.1 (−35.6 to −2.59)	8.31 (−3.58 to 20.2)	.047 *	10.9 (−4.79 to 26.6)	.058
Median (range)	−4.11 (−78.0 to 229.3)	−13.2 (−78.0 to 23.9)	−3.58 (−77.0 to 229.3)		−4.13 (−60.3 to 229.3)	
GCN change (≥1.0 units)	n (%)	n (%)	n (%)		n (%)	
Increase	16 (20.5)	0 (0.0)	15 (23.8)	.050	12 (27.3)	.059
Decrease	20 (25.6)	6 (30.0)	14 (22.2)		11 (25.0)	
No change	42 (53.8)	8 (19.0)	34 (54.0)		21 (47.7)	
GCN change (≥1.0 units)						
Increase	16 (20.5)	0 (0.0)	15 (23.8)	.059	12 (27.3)	.028 *
Decrease/no change	62 (79.5)	14 (100.0)	48 (76.2)		32 (72.7)	
GCN change (4.0 cutoff) [e]						
Increase	12 (15.4)	0 (0.0)	11 (17.5)	.047 *	9 (20.5)	.051
Decrease	16 (20.5)	6 (42.9)	10 (15.9)		8 (18.2)	
No change	50 (64.1)	8 (57.1)	42 (66.7)		27 (61.4)	
GCN change (4.0 cutoff) [e]						
Increase/no change	62 (79.5)	8 (57.1)	53 (84.1)	.062	35 (79.5)	.158
Decrease	16 (20.5)	6 (42.9)	10 (15.9)		9 (20.5)	

[a] Excluding 1 patient with missing SISH information from the primary tumor and 1 patient with pre–anti-EGFR treatment sample obtained from the metastatic site.

[b] Administered with or without chemotherapy after primary surgery but before obtaining the sample from the recurrent tumor. Excluding 1 patient with *KRAS*-mutated primary tumor.

[c] No anti-EGFR antibody treatment before obtaining the sample from the recurrent tumor.

[d] Mann-Whitney *U* test for continuous variables and Fisher exact test for categorical variables.

[e] GCN change from less than 4.0 to greater than 4.0 or vice versa.

* $P < .05$.

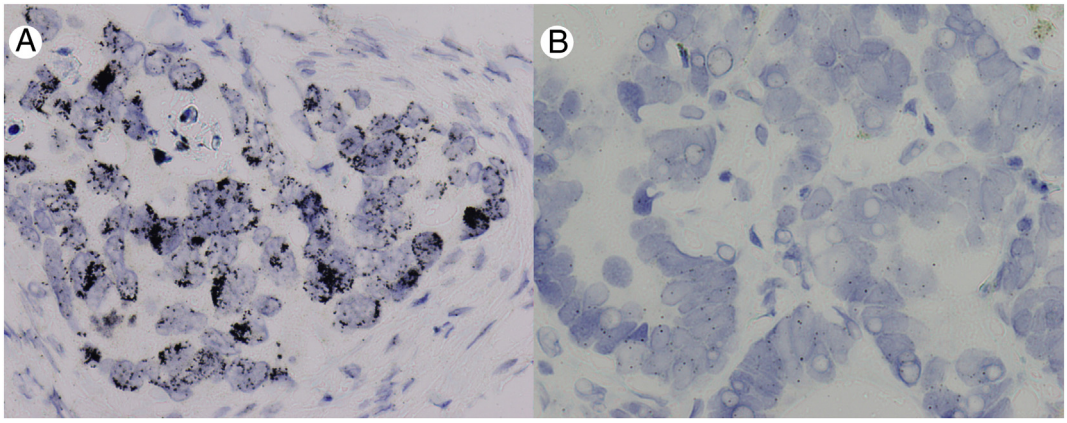

Figure EGFR GCN detected by SISH in primary colorectal adenocarcinoma and corresponding liver metastasis after anti-EGFR monoclonal antibody therapy. A, Primary tumor; average GCN, 15. B, Metastatic tumor; average GCN, 3.3. Original objective magnification ×40.

equivalent to a relative median change of −4.1% between the corresponding primary and recurrent tumors. The *EGFR* GCNs between the primary and recurrent tumors were not significantly different in the whole study population. When using *EGFR* GCN 4.0 as a cutoff, the GCN status did not significantly change during disease progression. The *EGFR* SISH results are presented in Table 2.

3.2. *EGFR* GCN decrease after anti-EGFR antibody therapy

EGFR GCN of the primary tumors did not differ between patients treated later with anti-EGFR therapy (n = 14) and patients receiving other forms of therapy (n = 63; Mann-Whitney *U* test, *P* = .588). Similarly, the *EGFR* GCN of the recurrent tumors did not differ between patients having received anti-EGFR therapy (n = 14) and patients not treated with anti-EGFR therapy (n = 64; Mann-Whitney *U* test, *P* = .123).

There was a significant decrease in *EGFR* GCN between the primary (mean, 5.55; median, 4.26) and recurrent tumors (mean, 3.92; median, 3.43) among the anti–EGFR-treated patients (Wilcoxon signed-rank test, *P* = .028) but not among patients not treated with anti-EGFR therapy. In addition, the relative GCN decrease was significantly more pronounced during anti-EGFR therapy than during other treatment regimens (Mann-Whitney *U* test, *P* = .047).

When *EGFR* GCN change of at least 1.0 was used as a cutoff value, GCN decrease or stable GCN was observed slightly more often among the anti–EGFR-treated patients than among patients not treated with anti-EGFR therapy (Fisher exact test, *P* = .050). None of the patients experienced GCN increase between the primary and recurrent tumors during anti-EGFR therapy. In contrast, *EGFR* GCN increased in 23.8% (15/63) of the patients during other treatment regimens. The association between *EGFR* GCN decrease and anti-EGFR therapy became more pronounced when including only patients with distant metastases in the analysis and using dichotomous classification (GCN increase versus decrease/no change; Fisher exact test, *P* = .028; relative risk, 1.38; 95% CI, 1.15-1.65).

With regard to the 4.0 cutoff value, discordant *EGFR* GCN was detected in 35.9% (28/78) of the primary-metastasis tumor

Table 4 Association of the *EGFR* GCN of the primary tumors with selected clinicopathological variables [a]

	n	*EGFR* GCN, median (range)	*P* [b]	*EGFR* GCN < 4.0, n (%)	*EGFR* GCN ≥ 4.0, n (%)	*P* [c]
Stage [d]						
Stages I-II	33	3.55 (2.50-10.0)	.024 *	21 (56.8)	12 (31.6)	.037 *
Stage III	42	4.25 (2.00-15.0)		16 (43.2)	26 (68.4)	
N						
N0	34	3.50 (2.50-10.0)	.018 *	22 (56.4)	12 (30.8)	.039 *
N1-2	44	4.25 (2.00-15.0)		17 (43.6)	27 (69.2)	
KRAS status						
Wild-type	45	4.20 (2.57-15.0)	.019 *	17 (43.6)	28 (71.8)	.021 *
Mutated	33	3.50 (2.00-8.00)		22 (56.4)	11 (28.2)	

[a] Excluding 1 patient with missing SISH information from the primary tumor and 1 patient with pre–anti-EGFR treatment sample obtained from the metastatic site.
[b] Mann-Whitney *U* test.
[c] Fisher exact test.
[d] Excluding 4 stage IV tumors.
* *P* < .05.

pairs among the whole study population. Among the anti–EGFR-treated patients, 42.9% (6/14) of the tumor pairs were discordant (McNemar test, $P = .031$), whereas among the patients not treated with anti-EGFR therapy (33.3% discordant pairs; 21/63), the GCN change was not significant (McNemar test, $P > .999$).

Table 3 compares the *EGFR* GCN alterations between patients treated with anti-EGFR antibodies and patients receiving other treatment regimens after primary surgery. *EGFR* GCN decrease between a primary colorectal adenocarcinoma and the corresponding liver metastasis after anti-EGFR antibody treatment is demonstrated in the Figure.

3.3. *EGFR* GCN in relation to the clinicopathological variables

Primary wild-type *KRAS* tumors had higher *EGFR* GCN (mean, 4.88; median, 4.20; range, 2.57-15.0) than did primary *KRAS*-mutated tumors (mean, 3.79; median, 3.50; range, 2.00-8.00; Mann-Whitney *U* test, $P = .019$). In *KRAS* wild-type tumors, *EGFR* GCN ≥ 4.0 was detected more often than in *KRAS*-mutated tumors (Fisher exact test, $P = .021$; OR, 3.29; 95% CI, 1.28-8.45).

EGFR GCN of the primary tumors was observed to be higher in patients with stage III disease (mean, 4.66; median, 4.25; range, 2.00-15.0) than in patients with stage I-II disease (mean, 3.96; median, 3.55; range, 2.50-10.0; Mann-Whitney *U* test, $P = .024$). Similarly, patients with stage III disease had more often *EGFR* GCN ≥ 4.0 than did patients with stage I-II disease (Fisher exact test, $P = .037$; OR, 2.84; 95% CI, 1.11-7.31). Particularly, patients with N1-2 lymph node status had higher *EGFR* GCN (mean, 4.79; median, 4.25; range, 2.00-15.0) than did patients with N0 (mean, 3.93; median, 3.50; range, 2.50-10.0; Mann-Whitney *U* test, $P = .018$). N1-2 lymph node status was also associated with *EGFR* GCN $\geq$ 4.0 (Fisher exact test, $P = .039$; OR, 2.91; 95% CI, 1.15-7.37).

No significant association was seen between *EGFR* GCN change and patient sex, age, the location of primary tumor (colon versus rectum or left-sided versus right-sided), the depth of tumor invasion, tumor differentiation grade, or recurrent tumor site (local versus distant). The results from selected clinicopathological analyses are shown in Table 4.

4. Discussion

Anti-EGFR treatment is currently recommended for the treatment for patients with *RAS* wild-type metastatic CRC [6,7]. We have previously shown that *EGFR* GCN, analyzed with SISH, is a promising predictive biomarker in these patients [8-10]. However, in previous studies, *EGFR* GCN analyses have mainly been performed on primary tumor tissue, and little is known about the potential effects of anti-EGFR therapy on *EGFR* GCN in recurrent disease [8-10,13,14]. In the present study, we have observed a decreasing trend in *EGFR* GCN among patients treated with anti-EGFR antibodies as compared with patients not receiving anti-EGFR therapy after primary surgery.

A few studies have investigated EGFR protein expression levels in primary and corresponding metastatic CRC tumors. However, data referring to *EGFR* GCN in metastatic tumors are limited, and few studies have compared *EGFR* GCN between primary and recurrent tumors in patients treated with anti-EGFR therapy. In these studies, discordance of *EGFR* GCN determined by fluorescent in situ hybridization has been reported in 5% to 13% of patients treated with anti-EGFR antibodies, but these analyses have not taken into account the *KRAS* status of the patients [15,16].

In a study by Molinari et al [17], 33% (12/36) of the patients were treated with anti-EGFR therapy. However, 5 of them were *KRAS* mutated and thus nonresponders. In line with our findings, the *EGFR* fluorescent in situ hybridization pattern remained stable between the primary and metastatic tumors in all of the *KRAS* wild-type patients treated with anti-EGFR antibodies, whereas 5 of the 24 patients not treated with anti-EGFR antibodies showed GCN increase.

In the present study, a significant decrease in *EGFR* GCN was observed between the primary and recurrent tumors among the anti–EGFR-treated patients but not among patients receiving other treatment regimens after primary surgery. Notably, none of the patients experiencing an *EGFR* GCN increase of at least 1.0 (19.5%; 15/77) were treated with anti-EGFR antibodies before obtaining the sample from the recurrent tumor. The association between anti-EGFR treatment and a GCN decrease of at least 1.0 did not reach statistical significance in the whole study population but became significant when including only patients with distant metastases in the analysis. This finding may be related to the overall accumulation of molecular changes being one of the mechanisms underlying the acquirement of metastatic capability of cancer cells [18]. Particularly, local CRC recurrences have been shown to be genetically more similar to the primary tumor than the distant metastases [19].

In addition, the proportion of tumors with *EGFR* GCN $<$ 4.0 increased significantly between the primary and recurrent tumors among the anti–EGFR-treated patients but not among patients receiving other treatment regimens. This change is interesting because it has been shown that *EGFR* GCN $\geq$ 4.0 in the primary tumor is associated with a favorable anti-EGFR antibody treatment response in metastatic disease [8-10]. Notably, none of the patients with tumor pairs showing *EGFR* GCN increase from less than 4.0 to at least 4.0 (14.3%; 11/77) received anti-EGFR antibodies before the sample was obtained from the recurrent tumor.

Our results show that *KRAS* wild-type primary tumors have higher *EGFR* GCN than *KRAS*-mutated tumors, which is in line with a previous report [20]. This is of interest because both (*K*)*RAS* wild-type status [2] and high *EGFR* GCN [8-10,13,21] are indicators of a beneficial response to EGFR-targeting antibodies in metastatic CRC. The RAS-RAF-MAPK signaling pathway is known to be constantly

active in *RAS*-mutated tumors, which contributes to resistance to anti-EGFR therapies [1]. The specific mechanism by which high *EGFR* GCN is associated with a favorable treatment response is still unknown. However, elevated *EGFR* GCN is observed to be related to Chr7 polysomy, and true gene amplifications are rare in CRC [8].

In our study, the number of patients treated with anti-EGFR therapy was too small for statistical analyses regarding the clinical response. Nevertheless, the patients who had a partial response to anti-EGFR therapy (8/13) had a mean *EGFR* GCN change of −2.43, whereas the patients with stable disease (4/13) had a mean GCN change of −0.82 and the 1 patient with progressive disease had a stable *EGFR* GCN.

It is possible that a selection pressure during the antibody treatment leads to the survival of cancer cells with smaller GCNs. Thus, it would be interesting to explore in a larger study population if a true correlation exists between *EGFR* GCN decrease and a favorable clinical treatment response from anti-EGFR antibodies. However, the study of tissue samples could be complicated by tumor heterogeneity, which is also known to affect *EGFR* GCN [9]. Few studies have been carried out investigating the *EGFR* GCN in metastatic tumors in relation to treatment response, but the number of patients has been too small to yield significant information [17].

Acquired resistance to anti-EGFR therapies is known to occur in a substantial proportion of patients, and several studies have been conducted to elucidate the mechanisms involved in this process [22-26]. It has been proposed that the antibody treatment functions as a selective pressure, which leads to the survival of those subclones that have genetic properties protecting them against the antibody [24]. Gene amplifications of other receptor tyrosine kinase genes such as *HER2* and *MET* have been noticed in patients who have acquired resistance to anti-EGFR antibodies. In addition, *KRAS* gene mutations and amplification as well as *NRAS* and *BRAF* mutations have been observed to associate with acquired antibody resistance [27-29]. Possible effects of *EGFR* GCN changes on acquired resistance have so far not been reported. However, it can be hypothesized that anti-EGFR antibody treatment could result in loss of EGFR polysomy in cancer cells and thereby contribute to acquired resistance.

In this study, lymph node–positive (stage III) tumors showed higher *EGFR* GCN than did lymph node–negative (stage I-II) tumors. Thus, increased *EGFR* GCN might denote the adenocarcinomas with higher invasive potential, which is in accordance with the known functions of EGFR signaling [1].

Although *EGFR* GCN was not found to be prognostic or predictive for overall survival in this study population (data not shown), its predictive value has been proven in other studies [8-10,13,21]. The present study was designed to compare *EGFR* GCN change between primary and recurrent disease, and our study material differs from the above-mentioned studies in some respects. In our study, the treatment for patients after recurrent disease was heterogeneous and only 30.0% of the patients were treated with EGFR-targeting antibodies. Among them, only 14 patients received anti-EGFR antibody treatment before obtaining the sample from the recurrent tumor. Our study material also included local recurrences (26.3%) in addition to distant metastases.

To conclude, this study suggests that anti-EGFR antibody treatment is associated with *EGFR* GCN decrease between the primary and corresponding recurrent colorectal adenocarcinomas, whereas no *EGFR* GCN change is observed in patients receiving other forms of treatment after primary surgery. These results warrant further investigation into a potential connection between *EGFR* GCN alterations and the evolution of clinical response during anti-EGFR treatment in advancing colorectal adenocarcinoma.

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