**EGFR** gene amplification is relatively common and associates with outcome in intestinal adenocarcinoma of the stomach, gastro-oesophageal junction and distal oesophagus

Eva-Maria Birkman\(^1\)*, Annika Ålgars\(^2,3\), Minnamaija Lintunen\(^1\), Raija Ristamäki\(^2\), Jari Sundström\(^1\) and Olli Carpén\(^1,4\)

### Abstract

**Background:** Approximately 50% of gastric adenocarcinomas belong to a molecular subgroup characterised by chromosomal instability and a strong association with the intestinal histological subtype. This subgroup typically contains alterations in the receptor tyrosine kinase–RAS pathway, for example **EGFR** or **HER2** gene amplifications leading to protein overexpression. In clinical practice, **HER2** overexpressing metastatic gastric cancer is known to respond to treatment with anti-**HER2** antibodies. By contrast, anti-**EGFR** antibodies have not been able to provide survival benefit in clinical trials, which, however, have not included patient selection based on the histological subtype or **EGFR** gene copy number analysis of the tumours. To examine the role of **EGFR** as a potential biomarker, we studied the prevalence, clinicopathological associations as well as prognostic role of **EGFR** and **HER2** expression and gene amplification in intestinal adenocarcinomas of the stomach, gastro-oesophageal junction and distal oesophagus.

**Methods:** Tissue samples from 220 patients were analysed with **EGFR** and **HER2** immunohistochemistry. Those samples with moderate/strong staining intensity were further analysed with silver *in situ* hybridization to quantify gene copy numbers. The results were associated with clinical patient characteristics and survival.

**Results:** Moderate/strong **EGFR** protein expression was found in 72/220 (32.7%) and **EGFR** gene amplification in 31/220 (14.1%) of the tumours, while moderate/strong **HER2** protein expression was detected in 31/220 (14.1%) and **HER2** gene amplification in 29/220 (13.2%) of the tumours. **EGFR** and **HER2** genes were co-amplified in eight tumours (3.6%). **EGFR** gene amplification was more common in tumours of distal oesophagus/gastro-oesophageal junction/cardia than in those of gastric corpus (\(p = 0.013\)). It was associated with shortened time to cancer recurrence (\(p = 0.026\)) and cancer specific survival (\(p = 0.033\)).

**Conclusions:** **EGFR** gene amplification is relatively common in intestinal adenocarcinomas and associates with decreased survival. It is rarely concurrent with **HER2** gene amplification, suggesting that anti-**EGFR** therapies might be applicable to some patients not eligible for anti-**HER2** treatment. Analogous to **HER2** testing, determination of **EGFR** gene amplification status in concert with immunohistochemistry could improve the specificity of patient selection when investigating the possible benefits of anti-**EGFR** therapies in the treatment of gastric adenocarcinomas.

**Keywords:** **EGFR**, **HER2**, Silver *in situ* hybridization, Gene amplification, Gastric cancer

---

*Correspondence:* emabir@utu.fi

\(^{1}\)Department of Pathology, University of Turku and Turku University Hospital, TYKS-SAPA, Turku, Finland

Full list of author information is available at the end of the article

© 2016 The Author(s). **Open Access** This article is distributed under the terms of the Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The Creative Commons Public Domain Dedication waiver (http://creativecommons.org/publicdomain/zero/1.0/) applies to the data made available in this article, unless otherwise stated.
Background
EGFR (ERBB1) and HER2 (ERBB2) are members of a tyrosine kinase receptor family frequently activated in cancer either by receptor overexpression or mutations. Metastatic HER2 overexpressing gastric or gastro-oesophageal junction (GOJ) adenocarcinomas can be treated with monoclonal anti-HER2 antibodies in combination with chemotherapy and the only targeted first-line antibody therapy for these tumours is trastuzumab. In contrast, monoclonal anti-EGFR antibodies are currently not indicated for the treatment of gastric cancer, although they are used for patients with metastatic colorectal or head and neck carcinomas.

Gastric adenocarcinomas are traditionally divided into intestinal and diffuse histological subtypes by Laurén classification [1]. Interestingly, it was recently suggested that these tumours can be classified into four distinct molecular subgroups based on their genomic alterations. One of the subgroups, characterised by chromosomal instability (CIN), accounts for about 50 % of gastric cancers and is strongly associated with the intestinal histological subtype and GOJ/cardiac location. Typical alterations in the CIN subtype include TP53 gene aberrations and activation of the receptor tyrosine kinase–RAS pathway, for example by receptor tyrosine kinase gene amplifications. In contrast, diffuse-type tumours are concentrated in a separate subgroup associating with overall genomic stability as well as distinctive genetic changes affecting cell adhesion and motility [2].

While anti-EGFR antibody treatment is beneficial in colorectal cancer [3, 4], no survival benefit has been observed in phase III clinical trials on gastric and gastro-oesophageal cancer for patients treated with anti-EGFR antibody-chemotherapy combination compared with patients treated with chemotherapy alone [5, 6]. Importantly, however, these studies included no patient selection based on the histological subtype of the tumours, EGFR protein expression or EGFR gene copy number (GCN) analysis. As demonstrated in the case of anti-HER2 therapy, an appropriate preselection with an easily applicable biomarker test might increase the potential to identify those patients who could benefit from anti-EGFR therapy.

In this study, we focused on intestinal adenocarcinomas in three locations: the stomach, gastro-oesophageal junction and distal oesophagus. Our aim was to examine the prevalence, clinicopathological associations as well as prognostic role of EGFR and HER2 protein expression and gene amplification in these tumours. First, we analysed EGFR and HER2 alterations by using immunohistochemistry (IHC) to select the tumours with moderate/strong expression of EGFR or HER2 protein. Second, we performed EGFR or HER2 silver in situ hybridisation (SISH) in selected cases to quantify GCNs. The validity of this algorithm for EGFR gene has previously been demonstrated with colorectal adenocarcinomas [7, 8] and was confirmed in this study by a set of control samples with negative or weak IHC staining.

Methods
Patients and clinical tumour material
The study population in this retrospective study consists of 220 patients diagnosed with intestinal adenocarcinoma of the stomach, gastro-oesophageal junction or distal oesophagus at the Turku University Hospital between the years 1993 and 2012. Initially, we used the clinical database of Auria Biobank (see below) to find all patients with the diagnosis of adenocarcinoma of the stomach, gastro-oesophageal junction or distal oesophagus (n = 437). The original histopathological information regarding these samples was then obtained to compile a preliminary list of patients, and the respective histological slides were retrieved from the archive. The exclusion criteria for this study were: diffuse or neuroendocrine histological subtype (n = 155), metastatic adenocarcinoma from a different organ (n = 6), intramucosal carcinoma (Tis) (n = 23) and insufficient sample material (n = 33). All cases were reanalysed by an expert gastrointestinal pathologist and the intestinal histological subtype of the tumours was confirmed by the presence of well-defined glandular structures in accordance with the Laurén classification [1]. Primarily, tissue samples from primary surgical specimens were included. In order to attain a comprehensive study population, representative biopsies were used in case of 22 patients (10 %): four (1.8 %) patients were not operated due to stage IV disease at the time of diagnosis and 18 (8.2 %) patients had received perioperative chemoradiotherapy resulting in insufficient surgical material for immunohistochemical analysis. The type of surgery was total gastrectomy for 120 (54.5 %) patients, subtotal gastrectomy or tumour resection for 79 (35.9 %) patients and palliative surgery for 17 (7.7 %) patients. The residual tumour classification was determined as R0 (no residual tumour) for 167 (75.9 %) patients, R1 (microscopic residual tumour) for 24 (10.9 %) patients and R2 (macroscopic residual) for 17 (7.7 %) patients. The residual tumour status could not be determined for 12 (5.5 %) patients. The median follow-up time for all patients was 10.5 years. The patient characteristics are presented in Table 1.

Tumour stage was assessed according to the current WHO Classification manual [9]. 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 histological samples were collected and analysed with the endorsement of the National Authority for Medico-Legal Affairs and The Ethics Committee of the Hospital District of Southwest Finland as well as with the permission of Auria Biobank.
|                                | Female, N (%) | Male, N (%) | All, N (%) |
|--------------------------------|---------------|-------------|------------|
| **Number of patients**         | 79 (35.9)     | 141 (64.1)  | 220        |
| **Age at diagnosis (years)**   |               |             |            |
| Median                         | 77            | 72          | 74         |
| Range                          | 33–93         | 43–90       | 33–93      |
| **Site of primary tumour**     |               |             |            |
| Distal oesophagus              | 4 (5.1)       | 16 (11.3)   | 20 (9.1)   |
| GOJ/cardia                     | 17 (21.5)     | 46 (32.6)   | 63 (28.6)  |
| Corpus                         | 21 (26.6)     | 44 (31.2)   | 65 (29.5)  |
| Antrum/pylorus                 | 37 (46.8)     | 35 (24.8)   | 72 (32.7)  |
| **Tumour differentiation grade**|             |             |            |
| Grade 1                        | 14 (17.7)     | 16 (11.3)   | 30 (13.6)  |
| Grade 2                        | 33 (41.8)     | 70 (49.6)   | 103 (46.8) |
| Grade 3                        | 32 (40.5)     | 55 (39.0)   | 87 (39.5)  |
| **Stage at diagnosis**         |               |             |            |
| I A                            | 15 (19.0)     | 18 (12.8)   | 33 (15.0)  |
| I B                            | 7 (8.9)       | 19 (13.5)   | 26 (11.8)  |
| IIA                           | 17 (21.5)     | 33 (23.4)   | 50 (22.7)  |
| IIB                           | 14 (17.7)     | 19 (13.5)   | 33 (15.0)  |
| IIIA                          | 7 (8.9)       | 21 (14.9)   | 28 (12.7)  |
| IIIB                          | 11 (13.9)     | 19 (13.5)   | 30 (13.6)  |
| IIIC                          | 1 (1.3)       | 5 (3.5)     | 6 (2.7)    |
| IV                            | 7 (8.9)       | 7 (5.0)     | 14 (6.4)   |
| **Residual tumour classification**|             |             |            |
| R0 (no residual tumour)        | 62 (78.5)     | 105 (74.5)  | 167 (75.9) |
| R1 (microscopic residual tumour) | 5 (6.3)     | 19 (13.5)   | 24 (10.9)  |
| R2 (macroscopic residual tumour) | 8 (10.1)   | 9 (6.4)     | 17 (7.7)   |
| Rx (unknown)                   | 4 (5.1)       | 8 (5.7)     | 12 (5.5)   |
| **Perioperative and adjuvant therapy** |          |             |            |
| Only chemotherapy              | 7 (9.7)       | 24 (17.9)   | 31 (15.0)  |
| Chemoradiotherapy              | 4 (5.6)       | 16 (11.9)   | 20 (9.7)   |
| Only radiation therapy         | 1 (1.4)       | 4 (3.0)     | 5 (2.4)    |
| No adjuvant therapy            | 58 (80.6)     | 89 (66.4)   | 147 (71.4) |
| Unknown                        | 2 (2.8)       | 1 (0.7)     | 3 (1.5)    |
| **Tumour recurrence**          |               |             |            |
| No recurrence                  | 55 (79.7)     | 82 (65.1)   | 137 (70.3) |
| Single metastasis >6 months    | 10 (14.5)     | 26 (20.6)   | 36 (18.5)  |
| Multiple metastases >6 months  | 4 (5.8)       | 18 (14.3)   | 22 (11.3)  |
| **Follow-up status**           |               |             |            |
| Alive and free of disease      | 22 (27.8)     | 31 (22.0)   | 53 (24.1)  |
| Alive with disease             | 1 (1.3)       | 1 (0.7)     | 2 (0.9)    |
| Died of disease                | 43 (54.4)     | 74 (52.5)   | 117 (53.2) |
| Died of other cause            | 12 (15.2)     | 30 (21.3)   | 42 (19.1)  |
| Unknown cause of death         | 1 (1.3)       | 5 (3.5)     | 6 (2.7)    |

GOJ gastro-oesophageal junction

"Excluding stage IV, "Excluding stage IV and recurrence <6 months"
hosting the specimen archive. All the specimens were from Auria biobank, which has obtained its archived diagnostic sample collection with an opt-out procedure according to the Finnish biobank act [10]. Biobanks authorized and inspected by National Supervisory Authority for Welfare and Health can provide human specimens collected during diagnostic procedures and associated clinical information for research purposes based on the biobank’s scientific board review. Thus, informed consent from surviving patients was not required.

Procedures

For each tumour, the most representative formalin-fixed paraffin-embedded (FFPE) tissue block was chosen and new sections were cut for both IHC staining and SISH. The methods for EGFR IHC and EGFR SISH have been described previously [7], and HER2 IHC was performed similarly with monoclonal HER2 antibody (clone 4B5, Ventana Medical Systems/Roche Diagnostics, Tucson, AZ, USA). HER2/Chr17 double-SISH was detected with HER2 DNA Probe and INFORM Chromosome 17 Probe (Ventana/Roche) and performed with ultraView SISH Detection Kit and ultraView Alkaline Phosphatase (AP) Red ISH Detection Kit (Ventana/Roche).

Immunohistochemistry and silver in situ hybridization

With EGFR, tumour scoring was based on the most intense membranous or membranous + cytoplasmic staining (0, negative; 1+, weak; 2+, moderate; 3+, strong). Strong staining was seen as intense reaction with 5x objective magnification, moderate staining was clearly identified with 5x objective magnification and weak staining was identified only with 10x objective magnification. Specimens were classified as IHC high if showing 2+ or 3+ membranous or membranous + cytoplasmic staining intensity in ≥10% of tumour cells in surgical specimens or in ≥5 clustered tumour cells in biopsies. These IHC high samples were further analysed with SISH. This algorithm is based on our previous observation that high EGFR IHC staining intensity positively correlates with increased EGFR GCN [7]. With HER2 IHC, tumours were scored according to standard criteria [11, 12] and specimens showing 2+ or 3+ membranous staining in ≥10% of tumour cells or in ≥5 clustered tumour cells in biopsies were classified as IHC high and analysed with SISH. EGFR and HER2 IHC and GCN were scored independently by two observers (EB and JS) without knowledge of the clinical information. Consensus scoring was used in case of differing individual results.

EGFR was quantified from the areas of high EGFR IHC intensity as described previously [7, 8]. Forty tumour cells with the highest number of copies were analysed from the EGFR SISH slides and an average value was calculated for each surgical sample. If these forty cells contained numerous overlapping EGFR SISH signals (clusters), the tumour was determined to have EGFR gene amplification. In biopsies, a group of ≥5 tumours cells with gene clusters was considered as amplification. One EGFR cluster was approximated to ≥10 gene copies. HER2 GCN was detected with chromosome 17 (Chr-17) number (number of copies of chromosome per cell) and the HER2/Chr-17 ratio was assessed according to standard criteria [13]. If HER2 gene clusters were detected in ≥10% of tumour cells in surgical specimens or in a group of ≥5 tumour cells in biopsies, the tumour was determined to contain HER2 gene amplification. One HER2 cluster was counted as ≥6 gene copies. To validate our method of including only tumours with high EGFR IHC intensity for EGFR SISH, we assessed EGFR GCN in fifteen randomly selected tumours in which EGFR IHC was scored as negative/weak. No EGFR amplification was found in these tumours (GCN 2.1–3.3).

Statistical analysis

Statistical analyses were performed with IBM SPSS Statistics for Windows, version 21.0 (IBM Corporation, Armonk, NY). Frequency table data were analysed using the χ² test, either with the Pearson χ² test or Fisher’s exact test for categorical variables. 2 × 2 tables were used to calculate odds ratios (OR). Kaplan-Meier method and log-rank test as well as Cox’s proportional hazards regression model were used for univariate survival analysis. Multivariate survival analysis was performed by Cox’s proportional hazards regression model. Variables with a p-value under 0.2 in univariate analysis were included in the multivariate analyses. Time to recurrence (TTR) was calculated from the time of diagnosis to the time of first recurrence, death of primary cancer or to the last follow-up date. Only recurrences occurring ≥6 months after diagnosis were considered relevant. Earlier detection of a local or distant recurrence was considered likely to present an initially advanced disease. Patients treated with surgery or surgery and adjuvant therapy without disease recurrence ≥6 months after diagnosis were considered relevant. Earlier detection of a local or distant recurrence was considered likely to present an initially advanced disease. Patients treated with surgery or surgery and adjuvant therapy without disease recurrence ≥6 months after diagnosis were considered curatively treated. Cancer-specific survival (CSS) was calculated from the time of diagnosis to the time of death of primary cancer or the last follow-up date and overall survival (OS) from the time of diagnosis to the time of death of any cause or the last follow-up date. Five patients (2.3%) who had received trastuzumab treatment for recurrent cancer were excluded from the CSS and OS analyses and additionally 14 patients with stage IV disease (6.4%) from the TTR analysis. All statistical tests were two-sided and p-values under 0.05 were considered statistically significant.
**Results**

**EGFR and HER2 immunohistochemical staining**
All 220 tumour samples were analysed with EGFR and HER2 IHC. High membranous or membranous + cytoplasmic EGFR IHC staining intensity (2+/3+) was observed in 72 (32.7 %) of the tumours, while 2+/3+ HER2 IHC staining intensity was present in 31 (14.1 %) tumours. Among these, concurrent high IHC staining intensity of EGFR and HER2 was detected in 14 (6.4 %) tumours. The results from EGFR and HER2 IHC stainings are shown in Table 2.

**EGFR and HER2 silver in situ hybridisation**
Gene copy numbers were analysed with EGFR or HER2 SISH in all tumours with high EGFR or HER2 IHC staining intensity. EGFR gene amplification was found in 31/72 tumours (14.1 % of the whole study material) and HER2 gene amplification in 29/31 tumours (93.5 % of the whole study material). Among these, EGFR and HER2 co-amplification was detected in 8/14 tumours (3.6 % of the whole study material). EGFR and HER2 gene amplification status was significantly concordant in antrum (Fisher’s exact test, p = 0.004). The results from EGFR and HER2 SISH stainings according to anatomical location are presented in Table 3. There was marked intratumoural heterogeneity of EGFR and HER2 gene amplification, as shown in Figs. 1 and 2.

**EGFR and HER2 protein expression and gene amplification in relation to clinicopathological variables**
Evaluated by IHC staining intensity, moderate or strong EGFR protein expression was associated with the depth of tumour invasion (pT3–pT4 versus pT1–pT2; Fisher’s exact test, p = 0.029); OR 2.15, 95 % CI: 1.11–4.17), but did not associate with tumour location (distal oesophagus/GOJ/cardia versus gastric corpus/antrum/pylorus; Fisher’s exact test, p = 0.054). In contrast, no significant association was found between HER2 protein expression levels and patient gender, tumour stage or histological differentiation grade.

**EGFR and HER2 gene amplification in relation to survival**
In univariate survival analysis, EGFR gene amplification was associated with shortened time to recurrence (TTR, median) (22 vs. 57 months, log-rank test, p = 0.026; Cox test, p = 0.028, HR: 1.73, 95 % CI: 1.06–2.83) and with shortened cancer-specific survival (CSS, median) (29 vs. 57 months, log-rank test, p = 0.033; Cox test, p = 0.035, HR: 1.67, 95 % CI: 1.04–2.69) (Fig. 3). Median TTR and CSS of the patients were both 45 months. HER2 gene amplification was not significantly associated with TTR, but patients with HER2 gene amplification had a notably lower median CSS of 22 months than patients without HER2 amplification (46 months). However, the difference was not statistically significant (log-rank test, p = 0.256) (Fig. 3).

In univariate analysis, increasing depth of tumour invasion was associated with decreased TTR and CSS.

**Table 2** Intensity of EGFR and HER2 immunohistochemical stainings in intestinal adenocarcinomas (N = 220)

| IHC staining intensity | EGFR, N (%) | HER2, N (%) | EGFR and HER2, N (%) |
|-----------------------|-------------|-------------|----------------------|
| 0/1+                  | 148 (67.3)  | 189 (85.9)  | 131 (59.5)           |
| 2+/3+                 | 72 (32.7)   | 31 (14.1)   | 14 (6.4)             |

*According to the most intense membranous or membranous + cytoplasmic staining

*According to the most intense membranous staining

*Concordant IHC staining intensity. In 75 tumours (34.1 %) IHC staining intensity was discordant
Similarly, increasing tumour stage was associated with decreased TTR and CSS (TTR: log-rank test, \( p < 0.0001 \); Cox test, \( p < 0.0001 \), HR 1.46, 95 % CI: 1.19–1.80 and CSS: log-rank test, \( p < 0.0001 \); Cox test, \( p < 0.0001 \), HR 1.60, 95 % CI: 1.30–1.96). This study shows that grade II and III tumours were associated with shorter TTR in comparison to grade I tumours (univariate Cox test, \( p = 0.043 \), HR 1.95, 95 % CI: 1.02–3.74). Additionally, grade II and III tumours were associated with shorter CSS in comparison to grade I tumours (univariate Cox test, grade II: \( p = 0.020 \), HR 2.22, 95 % CI: 1.13–4.36; grade III: \( p = 0.029 \), HR 2.15, 95 % CI: 1.08–4.27). No significant association was observed between EGFR or HER2 gene amplification status and overall survival (OS). EGFR or HER2 protein expression, evaluated by IHC staining intensity, was not significantly associated with TTR, CSS or OS.

In the multivariate model for TTR, EGFR gene amplification was analysed together with tumour stage, histological differentiation grade and tumour location. In the multivariate analysis for CSS, EGFR gene amplification was analysed together with tumour stage, histological differentiation grade and patient age at the time of diagnosis. Tumour stage remained as a single predictive factor for TTR (Cox test, stage III: \( p = 0.014 \), HR 2.05, 95 % CI: 1.16–3.63) as well as for CSS (Cox test, stage III: \( p = 0.023 \), HR 1.99, 95 % CI: 1.10–3.61; stage IV: \( p < 0.0001 \), HR 11.4, 95 % CI: 5.34–24.4). The results from univariate and multivariate survival analyses are presented in Table 5.

**Discussion**

This study shows that EGFR gene amplification is not uncommon in intestinal adenocarcinoma of the stomach, gastro-oesophageal junction and distal oesophagus. Furthermore, we demonstrate that EGFR amplification is most prevalent in proximally located tumours and significantly associated with decreased survival, as defined by TTR and CSS.

In previous studies, EGFR gene amplification has been reported to be present in only 2.3–4.9 % of gastric cancers including all histological subtypes [14–16], whereas the reported numbers for HER2 gene amplification vary between 7 and 17 % [17, 18]. The prevalence of EGFR and HER2 co-amplification has been reported as low (<0.5 %) [15, 16], albeit studies analysing concurrent EGFR and HER2 GCN changes are few and none have been carried out after the novel molecular subtypes of gastric cancer were published [2]. In contrast, we found EGFR gene amplification in 14.4 % and receptor co-amplification in 3.6 % of intestinal adenocarcinomas.
HER2 has been found to be overexpressed, as determined by both IHC and GCN analyses, in 7–25 % of gastric adenocarcinomas [11, 12, 17, 19] including all histological subtypes, which is comparable with our finding that high HER2 protein expression was found in 14.1 % and HER2 gene amplification in 13.2 % of intestinal adenocarcinomas.

Recent molecular classification studies have linked approximately 36–50 % of gastric adenocarcinomas
with characteristics such as intestinal-type histology, chromosomal abnormalities, changes in the receptor tyrosine kinase–RAS signaling pathway, as well as TP53 gene and somatic copy-number aberrations. These characteristics have been associated with a distinct molecular subgroup: tumours in the CIN subgroup are characterised by chromosomal instability, while the MSS/TP53\(^{-}\) subgroup typically contains microsatellite stable tumours with inactive TP53 [2, 20]. Both of these studies could further show that histologically diffuse-type tumours are concentrated in a separate subgroup with molecular characteristics different from those defining CIN or MSS/TP53\(^{-}\). However, the predominant anatomical location of tumours belonging to either CIN or MSS/TP53\(^{-}\) subgroup was found to differ: CIN tumours were mostly located in GOJ/cardia, whereas MSS/TP53\(^{-}\) tumours were predominantly situated in gastric antrum [2, 20]. It has been previously demonstrated that HER2 gene amplification is strongly associated with the intestinal histological subtype, as compared to the diffuse subtype, as well as with the gastro-oesophageal location of tumours [17, 19]. In our material, EGFR gene amplification was most common in the tumours of distal oesophagus and GOJ/cardia, as observed in the CIN subgroup, but infrequent in the tumours of gastric corpus. In antral/pyloric tumours, the observed prevalence of EGFR gene amplification was intermediate to that in other locations.

EGFR gene amplification was found to be significantly associated with decreased TTR and CSS, which is consistent with earlier findings of association between EGFR gene amplification and survival [14, 15]. Results from these studies are, however, based on notably smaller sample size and/or histologically more heterogeneous tumour material than included in this present study. There are contradictory reports regarding the relevance of HER2 gene amplification as a negative prognostic factor in gastric cancer [15, 17]. In this study, the non-significant association may partly be related to including only intestinal adenocarcinomas in the study material.

HER2 overexpression is known to predict treatment benefit from anti-HER2 antibody therapy. The survival of patients is significantly improved in metastatic gastric and gastro-oesophageal cancer by the addition of trastuzumab to a cisplatin-fluoropyrimidine-containing chemotherapy regimen [12], whereas no survival benefit has been

**Fig. 2** The association between strong EGFR/HER2 protein expression and EGFR/HER2 gene amplification in a single intestinal-type oesophagegoastric adenocarcinoma (original objective magnification 10x). All images are from the same area of the tumour: a Strong (3+) EGFR protein expression (IHC). b EGFR gene amplification (SISH). c Strong (3+) HER2 protein expression (IHC). d HER2 gene amplification (SISH). Insets show the gene amplification (original objective magnification 60x). Note that EGFR and HER2 are not amplified in the same cancer cells but in adjacent areas. IHC, immunohistochemistry; SISH, silver in situ hybridisation.
Table 4 Association between the clinicopathological variables and EGFR/HER2 protein expression or gene amplification (N = 220)

| Variable                        | EGFR IHC staining intensity, N (%) | EGFR in situ hybridisation status, N (%) | HER2 IHC staining intensity, N (%) | HER2 in situ hybridisation status, N (%) |
|---------------------------------|-----------------------------------|------------------------------------------|-----------------------------------|------------------------------------------|
|                                 | 0/1+ 2+/3+ | P value<sup>a</sup> | 0/1+ 2+/3+ | P value<sup>a</sup> | 0/1+ 2+/3+ | P value<sup>a</sup> | 0/1+ 2+/3+ | P value<sup>a</sup> |
| Patient gender                  |                                    |                                          |                                    |                                          |                                    |                                          |                                    |                                          |                                          |
| Female                          | 59 (39.9) | 20 (27.8) | NS    | 8 (25.8) | 71 (37.6) | NS    | 69 (36.5) | 10 (32.3) | NS    | 8 (27.6) | 71 (37.2) | NS    |                                          |                                          |                                          |                                          |                                          |                                          |                                          |                                          |                                          |                                          |
| Male                            | 89 (60.1) | 52 (72.2) | NS    | 23 (74.2) | 118 (62.4) | NS    | 120 (63.5) | 21 (67.7) | NS    | 21 (72.4) | 120 (62.8) | NS    |                                          |                                          |                                          |                                          |                                          |                                          |                                          |                                          |                                          |                                          |
| Site of primary tumour          |                                    |                                          |                                    |                                          |                                    |                                          |                                    |                                          |                                          |                                    |                                          |                                          |                                          |                                          |                                          |                                          |                                          |                                          |
| Distal oesophagus/GOJ/cardia    | 49 (33.1) | 34 (47.2) | NS    | 18 (58.1) | 65 (34.4) | 0.016 | 69 (36.5) | 14 (45.2) | NS    | 14 (48.3) | 69 (36.1) | NS    |                                          |                                          |                                          |                                          |                                          |                                          |                                          |                                          |                                          |
| Corpus/antrum/pylorus           | 99 (66.9) | 38 (52.8) | NS    | 13 (41.9) | 124 (65.6) | NS    | 120 (63.5) | 17 (54.8) | NS    | 15 (51.7) | 122 (63.9) | NS    |                                          |                                          |                                          |                                          |                                          |                                          |                                          |                                          |                                          |
| Histological differentiation grade |                                    |                                          |                                    |                                          |                                    |                                          |                                    |                                          |                                          |                                    |                                          |                                          |                                          |                                          |                                          |                                          |                                          |                                          |
| Grade I                         | 23 (15.5) | 7 (9.7) | NS    | 2 (6.5) | 28 (14.8) | NS    | 28 (14.8) | 2 (6.5) | NS    | 2 (6.9) | 28 (14.7) | NS    |                                          |                                          |                                          |                                          |                                          |                                          |                                          |                                          |                                          |
| Grade II                        | 71 (48.0) | 32 (44.4) | NS    | 17 (54.8) | 86 (45.5) | NS    | 83 (43.9) | 20 (64.5) | NS    | 18 (62.1) | 85 (44.5) | NS    |                                          |                                          |                                          |                                          |                                          |                                          |                                          |                                          |                                          |
| Grade III                       | 54 (36.5) | 33 (45.8) | NS    | 12 (38.7) | 75 (39.7) | NS    | 78 (41.3) | 9 (29.0) | NS    | 9 (31.0) | 78 (40.8) | NS    |                                          |                                          |                                          |                                          |                                          |                                          |                                          |                                          |                                          |
| Postoperative T<sup>b</sup>     |                                    |                                          |                                    |                                          |                                    |                                          |                                    |                                          |                                          |                                    |                                          |                                          |                                          |                                          |                                          |                                          |                                          |                                          |
| pT1–pT2                         | 54 (37.0) | 15 (21.4) | 0.029 | 4 (13.31) | 65 (34.9) | 0.020 | 63 (34.1) | 6 (19.4) | NS    | 6 (20.7) | 63 (33.7) | NS    |                                          |                                          |                                          |                                          |                                          |                                          |                                          |                                          |                                          |
| pT3–pT4                         | 92 (63.0) | 55 (78.6) | 26 (86.7) | 121 (65.1) | NS | 122 (65.9) | 25 (80.6) | NS | 23 (79.3) | 124 (66.3) | NS |                                          |                                          |                                          |                                          |                                          |                                          |                                          |                                          |                                          |
| Postoperative stage             |                                    |                                          |                                    |                                          |                                    |                                          |                                    |                                          |                                          |                                    |                                          |                                          |                                          |                                          |                                          |                                          |                                          |                                          |
| I–II                            | 100 (67.6) | 42 (58.3) | NS    | 14 (45.2) | 128 (67.7) | 0.024 | 125 (66.1) | 17 (54.8) | NS    | 15 (51.7) | 127 (66.5) | NS    |                                          |                                          |                                          |                                          |                                          |                                          |                                          |                                          |                                          |
| III–IV                          | 48 (32.4) | 30 (41.7) | 17 (54.8) | 61 (32.3) | 64 (33.9) | 14 (45.2) | 64 (33.5) | 14 (48.3) | 64 (33.5) |                                          |                                          |                                          |                                          |                                          |                                          |                                          |                                          |                                          |

<sup>a</sup>Fisher’s exact test

<sup>b</sup>N = 216, the depth of tumour invasion could not be determined for four patients not receiving surgical treatment
demonstrated in phase III clinical trials with anti-EGFR antibody treatment in comparison to other chemotherapeutic regimens [5, 6]. While the EGFR status was not used for patient selection in these earlier studies, an ongoing phase III clinical trial has been reported to select patients based on EGFR overexpression, although defined only by IHC [21]. Overexpression of EGFR protein has been reported in 24–27 % of all gastric adenocarcinomas [14, 16] and in 31 % [14] of intestinal gastric adenocarcinomas. In our study, we found that 32.7 % of the intestinal adenocarcinomas had high EGFR IHC staining intensity, but only 31/72 (43.1 %) of these demonstrated EGFR gene amplification. This suggests that determining EGFR overexpression only by IHC, without knowledge of the EGFR GCN, may be an inadequate method for selecting patients for anti-EGFR therapy. Indeed, a recent preclinical study with patient derived xenografts indicated that strongest response to anti-EGFR therapy was achieved in tumours with

**Fig. 3** Kaplan-Meier survival curves of intestinal-type oesophagogastric cancer patients with or without EGFR or HER2 amplification. Time to recurrence (a–b) and cancer-specific survival (c–d) as based on EGFR (a, c) and HER2 (b, d) SISH and IHC analyses. IHC, immunohistochemistry; SISH, silver in situ hybridisation
Table 5 Time to recurrence (TTR)\(^a\) and cancer-specific survival (CSS)\(^b\) of patients with intestinal-type adenocarcinomas

|                       | Univariate survival analysis for TTR | Multivariate survival analysis for TTR | Univariate survival analysis for CSS | Multivariate survival analysis for CSS |
|-----------------------|-------------------------------------|---------------------------------------|-------------------------------------|---------------------------------------|
|                       | Number of patients | TTR, median (months) | P value, log-rank test\(^c\) | P value, Cox test\(^d\) | HR | 95 % CI | Number of patients | CSS, median (months) | P value, log-rank test\(^a\) | P value, Cox test\(^b\) | HR | 95 % CI |
| Age (continuous variable) | 198 | NS | | | | | 212 | 21.2 | NS | | | |
| Patient gender | | | | | | | | | | | | | |
| Female (reference) | 71 | 56.6 | NS | | | | 78 | 45.6 | NS | | | |
| Male | 127 | 38.2 | | | | | 134 | 44.6 | | | | |
| Site of primary tumour | | | | | | | | | | | | | |
| Distal oesophagus/GOJ cardia (reference) | 75 | 28.5 | NS | | | | 77 | 34.3 | NS | | | |
| Corpus/antrum/pylorus | 123 | 53.6 | NS | | | | 135 | 47.1 | NS | | | |
| Histological differentiation grade | | | | | | | | | | | | | |
| Grade I (reference) | 30 | NA | NS | | | | 30 | NA | NS | | | |
| Grade II | 92 | 33.8 | 0.043 | 1.95 | 1.02–3.74 | NS | 98 | 33.8 | 0.020 | 2.22 | 1.13–4.36 | NS | |
| Grade III | 76 | 53.2 | NS | | | | 84 | 44.6 | 0.029 | 2.15 | 1.08–4.27 | NS | |
| Postoperative T | | | | | | | | | | | | | |
| pT1 (reference) | 37 | 67.3 | <0.0001 | | | | 37 | NA | <0.0001 | | | |
| pT2 | 31 | NA | NS | | | | 31 | NA | NS | | | |
| pT3 | 75 | 56.6 | NS | | | | 82 | 57.3 | NS | | | |
| pT4 | 55 | 20.5 | 0.002 | 2.59 | 1.44–4.67 | | 59 | 25.7 | 0.001 | 2.94 | 1.58–5.47 | |
| Postoperative stage | | | | | | | | | | | | | |
| I (reference) | 58 | NA | 0.005 | | | | 58 | NA | <0.0001 | | | |
| II | 80 | 38.2 | NS | | | | 80 | 57.3 | NS | | | |
| III | 60 | 22.6 | 0.001 | 2.33 | 1.38–3.92 | 0.014 | 2.05 | 1.16–3.63 | 60 | 29.0 | 0.002 | 2.36 | 1.37–4.08 | 0.023 | 1.99 | 1.10–3.61 | |
| IV | NA | | | | | | 14 | 6.90 | <0.0001 | 14.2 | 6.86–29.3 | <0.0001 | 11.4 | 5.34–24.4 | |
| EGFR amplification | | | | | | | | | | | | | |
| Yes | 28 | 21.8 | 0.026 | 0.028 | 1.73 | 1.06–2.83 | NS | 30 | 29.0 | 0.033 | 0.035 | 1.67 | 1.04–2.69 | NS | |
| No (reference) | 170 | 56.6 | | | | | 182 | 57.3 | | | | |
Table 5 Time to recurrence (TTR)\textsuperscript{a} and cancer-specific survival (CSS)\textsuperscript{b} of patients with intestinal-type adenocarcinomas (Continued)

| HER2 amplification | TTR (yrs) | CSS (yrs) | p-value | Hazard ratio |
|--------------------|-----------|-----------|---------|--------------|
| Yes                | 20        | 44.6      | NS      | NS           |
| No (reference)     | 178       | 45.6      | 25      | 22.3         |

NS not significant, NA not applicable
\(\textsuperscript{a}\)Excluding trastuzumab-treated and stage IV patients
\(\textsuperscript{b}\)Excluding trastuzumab-treated patients
\(\textsuperscript{c}\)Kaplan-Meier method
\(\textsuperscript{d}\)Cox's proportional hazards regression model
EGFR gene amplification [22]. The relatively low prevalence of co-amplification of EGFR and HER2 genes (3.6 % in this study) demonstrates the presence of two distinct subgroups of patients with either EGFR or HER2 gene amplification, which implies that anti-EGFR therapies might be applicable to some patients not eligible for anti-HER2 treatment. Those patients having receptor co-amplification might even benefit from a dual-acting antibody treatment.

Conclusions
In this study, we have shown that EGFR gene amplification is relatively common in intestinal adenocarcinomas of the stomach, gastro-oesophageal junction and distal oesophagus and associates with decreased survival. We have also demonstrated that EGFR GCN can be easily analysed by silver in situ hybridisation in diagnostic tumour material and thus could be applied as a routine histopathological diagnostic method. Based on our results, we suggest that determining EGFR gene amplification status in concert with IHC could be used in future clinical trials to identify patients with inverse prognosis and to improve the specificity of patient selection when investigating the possible benefits of anti-EGFR therapies in the treatment of intestinal-type gastro-oesophageal adenocarcinomas.

Abbreviations
Chr-17, chromosome 17; CI, confidence interval; CIN, chromosomal instability; CSS, cancer-specific survival; EGFR (ERBB1), epidermal growth factor receptor (erb-b2 receptor tyrosine kinase 1); FFPE, formalin-fixed paraffin-embedded; GCN, gene copy number; GOJ, gastro-oesophageal junction; HER2 (ERBB2), human epidermal growth factor receptor 2 (erb-b2 receptor tyrosine kinase 2); HR, hazard ratio; IHC, immunohistochemistry; MSS, microsatellite stable; NA, not applicable; NS, not significant; OR, odds ratio; OS, overall survival; pT, pathologic T, describes tumour size and depth of invasion; RAS, rat sarcoma viral oncogene homolog; SISH, silver in situ hybridization; TP53, tumour protein p53; TTR, time to recurrence.

Acknowledgements
We thank Ms Susanna Hussi, Ms Jaana Nurminen and Ms Katja Tamminen for collecting the archived FFPE samples, Ms Sinikka Kollanus and Ms Paula Merilähti for preparing the tissue sections for IHC and SISH and Mr Jaakko Liippo for help with the pictures.

Funding
This study was supported by the Turku University Foundation, the Cancer Society of Southwest Finland and the Special Government Funding (EVO) allocated to Turku University Hospital. The funders had no role in study design, data collection, analysis and interpretation, decision to publish or preparation of the manuscript.

Availability of data and materials
All supporting data for the conclusions are included within the article.

Authors’ contributions
EB collected the clinicopathological data, contributed to the histological and molecular analysis, performed the statistical analysis, contributed to data analysis and drafted the manuscript. AÅ contributed to statistical analysis, clinical interpretation and manuscript preparation. ML coordinated the molecular testing and contributed to data analysis and manuscript preparation. RR contributed to the collection of clinical data, clinical interpretation and manuscript preparation. JS coordinated the sample choice and provision, verified the histological analysis and contributed to the molecular analysis and manuscript preparation. OC contributed to the overall study design, data interpretation and manuscript preparation. All authors have read and approved the final manuscript.

Competing interests
AÅ, ML, RS, JS and OC are inventors in a patent related to this work: US 20110217296 A1 Method for selecting patients for treatment with an EGFR inhibitor. AÅ has had an advisory role with Lilly. All remaining authors declare that they have no competing interests.

Consent for publication
Not applicable.

Ethics approval and consent to participate
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 histological samples were collected and analysed with the endorsement of the National Authority for Medicco-Legal Affairs and The Ethics Committee of the Hospital District of Southwest Finland as well as with the permission of Auria Biobank hosting the specimen archive. All the specimens were from Auria biobank, which has obtained its archived diagnostic sample collection with an opt-out procedure according to the Finnish biobank act. Biobanks authorized and inspected by National Supervisory Authority for Welfare and Health can provide human specimens collected during diagnostic procedures and associated clinical information for research purposes based on the biobank’s scientific board review. Thus, informed consent from surviving patients was not required.

Author details
1Department of Pathology, University of Turku and Turku University Hospital, TYKS-SAPA, Turku, Finland. 2Department of Oncology, University of Turku and Turku University Hospital, Turku, Finland. 3MedCity Research Laboratory, University of Turku, Turku, Finland. 4Auria Biobank, Turku, Finland.

Received: 27 October 2015 Accepted: 23 June 2016
Published online: 07 July 2016

References
1. Lauren P. The two histological main types of gastric carcinoma: diffuse and so-called intestinal-type carcinoma. An attempt at a histo-clinical classification. Acta Pathol Microbiol Scand. 1965;64:31–49.
2. Cancer Genome Atlas Research Network. Comprehensive molecular characterization of gastric adenocarcinoma. Nature. 2014;513:202–9.
3. Bokemeyer C, Bondarenko I, Makhson A, Hartmann JT, Aparicio J, De Braud F, Donea S, Ludwig H, Schuch G, Stroh C, et al. Fluorouracil, leucovorin, and oxaliplatin with and without cetuximab in the first-line treatment of metastatic colorectal cancer. J Clin Oncol. 2009;27:663–71.
4. Douillard J-Y, Siena S, Cassidy J, Tabernero J, Burkes R, Barugel M, Humbert Y, Bodoky G, Cunningham D, Jassem J, et al. Randomized, Phase III trial of panitumumab with infusional fluorouracil, leucovorin, and oxaliplatin (FOLFOX4) Versus FOLFOX4 alone as first-line treatment in patients with previously untreated metastatic colorectal cancer: The PRIME study. J Clin Oncol. 2010;28:4697–705.
5. Lordick F, Kang Y-K, Chung H-C, Salaman P, Oh SC, Bodoky G, Kurtova G, Volovat C, Moisyuenko VM, Gorbunova V, et al. Capecitabine and cisplatin with or without cetuximab in patients with previously untreated advanced gastric cancer (EXPAND): A randomised, open-label phase 3 trial. Lancet Oncol. 2013;14:490–9.
6. Waddeel T, Chau I, Cunningham D, Gonzalez D, Frances A, Okines C, Wotherspoon A, Saffery C, Middleton G, Waddley J, et al. Epirubicin, oxaliplatin, and capcitabine with or without panitumumab for patients with previously untreated advanced gastric cancer (EXPAND): A randomised, open-label phase 3 trial. Lancet Oncol. 2013;14:481–9.
7. Ålgars A, Lintunen M, Carpén O, Ristimäki R, Sundström J. EGFR gene copy number assessment from areas with highest EGFR expression predicts response to anti-EGFR therapy in colorectal cancer. Br J Cancer. 2011;105:255–62.
8. Ålgars A, Avoranta T, Österlund P, Lintunen M, Sundström J, Jokilehto T, Ristimäki A, Ristimäki R, Carpén O. Heterogeneous EGFR gene copy number increase is common in colorectal cancer and defines response to anti-EGFR therapy. PLoS One. 2014;9(6):e99590.
9. Bosman F, Carneiro F, Harris H, Hruban R, Theise N, editors. WHO Classification of Tumours of the Digestive System. 4th ed. Lyon: IARC Press; 2010.

10. Finlex Data Bank. https://www.finlex.fi/fi/laki/kaannokset/2012/en20120688.pdf. Accessed 26 October 2015.

11. Hofmann M, Stoss O, Shi D, Büttner R, Van De Vijver M, Kim W, Ochiai A, Rüschoff J, Henkel T. Assessment of a HER2 scoring system for gastric cancer: results from a validation study. Histopathology. 2008;52:797–805.

12. Bang Y-J, Van Cutsem E, Feyereislova A, Chung HC, Shen L, Sawaki A, Lordick F, Ohtsu A, Omuro Y, Satoh T, et al. Trastuzumab in combination with chemotherapy versus chemotherapy alone for treatment of HER2-positive advanced gastric or gastro-oesophageal junction cancer (ToGA): A phase 3, open-label, randomised controlled trial. Lancet. 2010;376:687–97.

13. Grogan TM, McElhinny AS, Loftin IR, Warren SL, Sugarman M, Miller R, Olivas-Brochu E, Roche P, Walk E, Padilla M, et al. Interpretation Guide. Ventana INFORM HER2 Dual ISH DNA Probe Cocktail Assay. 2010. http://www.uclad.com/newsletters/HER2_DDISH_Interpretation_Guide.pdf. Accessed 4 July 2016.

14. Kim MA, Lee HS, Lee HE, Jeon YK, Yang HK, Kim WH. EGFR in gastric carcinomas: Prognostic significance of protein overexpression and high gene copy number. Histopathology. 2008;52:738–46.

15. Kandel C, Leclair F, Bou-Hanna C, Laboisse CL, Mosnier J-F. Association of HER1 amplification with poor prognosis in well differentiated gastric carcinomas. J Clin Pathol. 2014;67:307–12.

16. Nagatsuka AK, Azawia M, Kuswara T, Doi T, Ohtsu A, Fuji H, Ochiai A. Expression profiles of HER2, EGFR, MET and FGFR2 in a large cohort of patients with gastric adenocarcinoma. Gastric Cancer. 2015;18:227–38.

17. Tanner M, Holliën M, Junttila TT, Kapanen AI, Tommola S, Soini Y, Helin H, Salo J, Joensuu H, Silhvo E, et al. Amplification of HER-2 in gastric carcinoma: Association with Topoisomerase Ila gene amplification, intestinal type, poor prognosis and sensitivity to trastuzumab. Ann Oncol. 2005;16:273–8.

18. Takehana T, Kunitomo K, Kono K, Kihara F, Izuka H, Matsumoto Y, Fujino MA, Doi A. Status of c-erbB-2 in gastric adenocarcinoma: a comparative study of immunohistochemistry, fluorescence in situ hybridization and enzyme-linked immuno-sorbent assay. Int J Cancer. 2002;98:833–7.

19. Gravalos C, Jimeno A. HER2 in gastric cancer: A new prognostic factor and a novel therapeutic target. Ann Oncol. 2008;19:1523–9.

20. Cristescu R, Nebozhy M, Kim K-M, Ting J, Wong S, Liu J, Yue Y, Wang J, Yu K, Ye X, et al. Molecular analysis of gastric cancer identifies subtypes associated with distinct clinical outcomes. Nat Med. 2015;21:449–56.

21. Kuhnl Pharmaceutical Co. L. Phase 3 Study of Nimotuzumab and Irinotecan as Second Line With Advanced or Recurrent Gastric and Gastroesophageal Junction Cancer. Bethesda: National Library of Medicine; 2013. https://clinicaltrials.gov/ct2/show/NCT01812252. Accessed 08 Oct 2015.

22. Zhang L, Yang J, Cai J, Song X, Deng J, Huang X, Chen D, Yang M, Very J-P, Li S, et al. A subset of gastric cancers with EGFR amplification and overexpression respond to cetuximab therapy. Sci Rep. 2013;3:2992.