



**HAL**  
open science

## ERBB2 Comprehensive Profiling and Prognostication in Stage III Colon Cancer: Findings From PETACC8 and IDEA-France Cohorts

Camilla Pilati, Audrey Soulabaille, Claire Gallois, H el ene Blons, Anne Cayre, Marine Sroussi, Delphine Le Corre, Sophie Mouillet-Richard, Claire Mulot, Karine Le Malicot, et al.

► **To cite this version:**

Camilla Pilati, Audrey Soulabaille, Claire Gallois, H el ene Blons, Anne Cayre, et al.. ERBB2 Comprehensive Profiling and Prognostication in Stage III Colon Cancer: Findings From PETACC8 and IDEA-France Cohorts. *Gastroenterology*, 2024, 10.1053/j.gastro.2024.10.046 . hal-04955612

**HAL Id: hal-04955612**

**<https://hal.science/hal-04955612v1>**

Submitted on 21 Feb 2025

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destin ee au d ep ot et  a la diffusion de documents scientifiques de niveau recherche, publi es ou non,  emanant des  tablissements d'enseignement et de recherche fran ais ou  trangers, des laboratoires publics ou priv es.

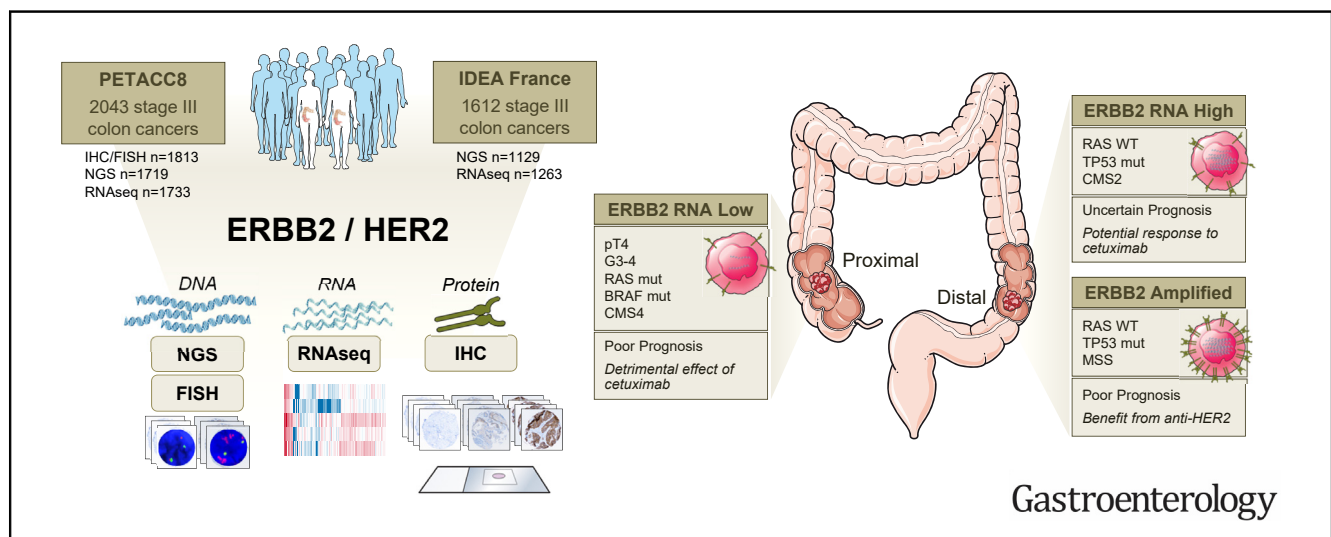


Distributed under a Creative Commons Attribution 4.0 International License

# ERBB2 Comprehensive Profiling and Prognostication in Stage III Colon Cancer: Findings From PETACC8 and IDEA-France Cohorts

Camilla Pilati,<sup>1</sup> Audrey Soulabaille,<sup>1</sup> Claire Gallois,<sup>1,2</sup> H el ene Blons,<sup>1,2</sup> Anne Cayre,<sup>3</sup> Marine Sroussi,<sup>1,4</sup> Delphine Le Corre,<sup>1</sup> Sophie Mouillet-Richard,<sup>1</sup> Claire Mulo t,<sup>1</sup> Karine Le Malicot,<sup>5</sup> Aur elien De Reynies,<sup>1</sup> Jean-Baptiste Bachet,<sup>1,6,7</sup> Christophe Borg,<sup>8</sup> Fr ed eric Di Fiore,<sup>9</sup> Rosine Guimbaud,<sup>10</sup> Jaafar Bennouna,<sup>11</sup> Thierry Andr e,<sup>12</sup> Julien Taieb,<sup>1,2,7</sup> Fr ed erique Penault-Llorca,<sup>3</sup> and Pierre Laurent-Puig<sup>1,2</sup>

<sup>1</sup>Centre de Recherche des Cordeliers, Sorbonne Universit e, Institut National de la Sant e et de la Recherche M edicale, Universit e Paris Cit e, Personalized Medicine, Pharmacogenomics and Therapeutic Optimization, Paris, France; <sup>2</sup>Institut du Cancer Paris, Cancer Research for Personalized Medicine, Assistance Publique–H opitaux de Paris Centre, H opital Europ een Georges Pompidou, Paris, France; <sup>3</sup>Department of Pathology, Centre Jean Perrin, UNICANCER, Institut National de la Sant e et de la Recherche M edicale, UMR 1240, Imagerie Mol eculaire et Strat egies Th eranostiques, University Clermont Auvergne, Clermont-Ferrand, France; <sup>4</sup>Institut Chimie Biologie Innovation, Laboratoire de BioChimie,  cole Sup erieure de Physique et de Chimie Industriel, UMR 8231, Centre National de la Recherche Scientifique, Paris Sciences et Lettres University, Paris, France; <sup>5</sup>F ed eration Francophone de Canc erologie Digestive, Epid emiologie et Recherche Clinique en Canc erologie Digestive, Institut National de la Sant e et de la Recherche M edicale, Lipides Nutrition Cancer, UMR 1231, University of Burgundy and Franche Comt e, Dijon, France; <sup>6</sup>Department of Hepato-Gastroenterology, Groupe Hospitalier Piti e Salp etri ere, Paris, France; <sup>7</sup>Association des Gastroent erologues Oncologues, Paris, France; <sup>8</sup>Department of Medical Oncology, University Hospital of Besan on, Besan on, France; <sup>9</sup>Hepatogastroenterology Department, Centre Hospitalier Universitaire Rouen, University of Rouen Normandy, Institut National de la Sant e et de la Recherche M edicale 1245, Institut de Recherche en Oncologie Group, Normandie University, Rouen, France; <sup>10</sup>Digestive Medical Oncology, Centre Hospitalier Universitaire de Toulouse, Toulouse, France; <sup>11</sup>Thoracic Oncology Unit, University Hospital of Nantes and Institut National de la Sant e et de la Recherche M edicale, Regional Center for Research in Cancerology and Immunology, Nantes, France; and <sup>12</sup>Sorbonne Universit e and H opital Saint Antoine, Institut National de la Sant e et de la Recherche M edicale 938, Site de Recherche Int egr ee sur le Cancer Curamus, Paris, France



**BACKGROUND & AIMS:** ERBB2 pathway activation, through amplification or activating mutations, represents a new target for colon cancer (CC) treatment. Molecular methods were compared with the gold standard for assessing ERBB2 status, and the prognostic value of ERBB2 amplification, mutations, and expression was determined using data from 2 phase 3 trials involving nearly 3000 patients with stage III CC. **METHODS:** In the PETACC8 trial, immunohistochemistry and fluorescence in situ hybridization, DNA, and RNA analysis were

performed on 1813, 1719, and 1733 samples, respectively. In the IDEA-France trial, DNA and RNA sequencing was performed on 1129 and 1263 samples. The breast cancer SCAN-B cohort (N = 3409) served as an external reference. A new molecular ERBB2-amplified status was defined using ERBB2 next-generation sequencing score, RNA sequencing expression, and clustering based on ERBB2 neighboring gene expression. Concordance between diagnostic techniques and the association between time to recurrence (TTR) and ERBB2-status were

evaluated. **RESULTS:** The prevalence of the molecular ERBB2-amplified group was 1.85% in PETACC8 and 1.5% in IDEA-France, with a concordance of 0.81 (95% CI, 0.70–0.92) with the gold standard immunohistochemistry and fluorescence in situ hybridization method in PETACC8. A nonlinear relationship was observed between TTR and ERBB2 expression, with extreme groups showing a less favorable prognosis ( $P < .0001$ ) in both colon and breast cancers. Patients with molecular ERBB2-amplified status or mutations had the poorest prognosis, followed by low-expression and intermediate-expression groups (3-year TTR of 67.0%, 71.2%, and 77.9%, respectively). In multivariate analysis, the low-expression group had a significantly shorter TTR (hazard ratio, 1.28; 95% CI, 1.07–1.52). **CONCLUSIONS:** The molecular definition of ERBB2 status could represent a cost-effective alternative in stage III CC. ERBB2 alterations and low RNA expression significantly reduced TTR, highlighting the complex role of ERBB2.

**Keywords:** HER2 Amplification; ERBB2 Expression; IHC/FISH; RNA-seq; NGS Score; Stage III CC Prognosis.

Colon cancer (CC) is a major public health concern, ranking third in cancer incidence and second in cancer-related deaths.<sup>1</sup> Stage III localized CC, constituting approximately 35% of diagnoses, presents heterogeneous prognoses, with 5-year overall survival rates ranging from 45% to 65%.<sup>2</sup>

The *ERBB2* gene, also known as HER2, encodes a tyrosine kinase receptor belonging to the epidermal growth factor receptor (EGFR) family. The amplification of *ERBB2* and the subsequent overexpression of the HER2 protein are observed in 15%–30% of breast cancers and 10%–30% of gastric cancers. This overexpression is a poor prognostic marker that is effectively targeted by anti-HER2 therapy.<sup>3</sup> ERBB2 amplification occurs in 5% of metastatic colorectal cancer cases with *RAS* wild type and 2%–3% of cases regardless of *RAS* status, yet its prognostic and predictive significance remains uncertain.<sup>4–7</sup> Somatic activating mutations in ERBB2, which occasionally occur alongside amplification, lack clear prognostic data, but suggest potential therapeutic relevance.<sup>4–6,8–12</sup> Results of phase 2 studies testing dual anti-HER2 inhibition in ERBB2-amplified metastatic colorectal cancer showed promise in heavily pretreated patients,<sup>10,11,13</sup> emphasizing the need for further research into their predictive and prognostic value, particularly in stage III CC, where adjuvant strategies require improvement.

Currently, the gold standard for assessing *ERBB2* amplification status in CC involves immunohistochemistry (IHC), followed by fluorescence in situ hybridization (FISH) in ambiguous cases. Although widely established and used in the clinical setting, these techniques rely on pathologists' expertise. Meanwhile, "omic" methods, particularly those leveraging next-generation sequencing (NGS), have become increasingly popular for detailed examination of the tumor's molecular profile at both DNA and RNA levels. This study aims to identify surrogate markers for *ERBB2* amplification through RNA and DNA analyses, comparing them with established methods. Using data from the prospective

## WHAT YOU NEED TO KNOW

### BACKGROUND AND CONTEXT

Activation of the ERBB2/HER2 pathway in colon cancer presents a critical opportunity for targeted therapies, necessitating accurate methods for assessing ERBB2 status.

### NEW FINDINGS

This study introduces a novel molecular definition of ERBB2 amplification and highlights the nonlinear relationship between survival and ERBB2 expression in both colon and breast cancers.

### LIMITATIONS

Clinical trials investigating anti-HER2 therapies as neoadjuvant treatments in stage III colon cancer are currently underway, which may limit the immediate applicability of these findings in clinical practice.

### CLINICAL RESEARCH RELEVANCE

Understanding ERBB2 status is crucial for effectively stratifying patients, as those with ERBB2 amplification or low RNA expression tend to have poorer prognoses and exhibit differing responses to anti-epidermal growth factor receptor treatments. This knowledge can guide treatment decisions and inform future clinical trials.

### BASIC RESEARCH RELEVANCE

This study enhances the foundational understanding of ERBB2's role in colorectal cancer biology, providing insights that may lead to innovative therapeutic strategies.

PETACC8 and IDEA-France studies, we analyzed concordance among techniques (IHC, FISH, NGS, and RNA sequencing [RNA-seq]) in assessing ERBB2 genomic amplification and its impact on RNA and protein expressions, ultimately determining its prognostic value in stage III CC.

## Methods

### Patient Population

The PETACC8 trial, a phase 3 randomized study, enrolled 2559 patients with stage III CC comparing the FOLFOX4 (5-fluorouracil, leucovorin, and oxaliplatin) regimen with FOLFOX4 plus cetuximab in the adjuvant setting.<sup>14</sup> Among them, 2043 patients consented to participate in translational research. Of these, 1813 (88.7%) had samples for immunohistochemistry and FISH analyses. Tumor DNA and RNA were available for 1719 patients (84.1%) and 1733 patients (84.8%).

IDEA-France, a part of the IDEA international collaboration, is a phase 3 randomized trial involving 2000 patients with stage III

**Abbreviations used in this paper:** CC, colon cancer; CMS, consensus molecular subtype; EGFR, epidermal growth factor receptor; FFPE, formalin-fixed, paraffin-embedded; FISH, fluorescence in situ hybridization; FOLFOX4, oxaliplatin, leucovorin, and 5-fluorouracil; HR, hazard ratio; IHC, immunohistochemistry; NGS, next-generation sequencing; RNA-seq, RNA sequencing; TTR, time to recurrence.

© 2024 The Author(s). Published by Elsevier Inc. on behalf of the AGA Institute. This is an open access article under the CC BY license (<http://creativecommons.org/licenses/by/4.0/>).

0016-5085

<https://doi.org/10.1053/j.gastro.2024.10.046>

CC, comparing 3–6 months of oxaliplatin-based adjuvant treatment (modified FOLFOX6 or CAPOX [capecitabine plus oxaliplatin]).<sup>15</sup> Among these patients, 1612 consented to participate in translational research. Tumor DNA and RNA were available for 1129 patients (70.0%) and 1263 patients (78.3%). ERBB2 status was not assessed by IHC and FISH in the IDEA-France population.

The SCAN-B cohort (GSE96058 dataset; N = 3409; Illumina HiSeq 2000) was used as an external cohort.<sup>16</sup>

## Molecular Analysis

**ERBB2 immunohistochemistry and fluorescence in situ hybridization status.** ERBB2 IHC and FISH were conducted on tissue microarrays. Tissue microarrays were generated with a 2-mm punch size from macrodissected formalin-fixed, paraffin-embedded (FFPE) blocks from the primary tumor after deparaffinization and rehydration. HER2/ERBB2 IHC staining was performed on fresh sections, cut within 24 hours, using the anti-HER2/neu monoclonal antibody (Clone: 4B5 Ventana predilution; Roche Diagnostics, Rotkreuz, Switzerland) on tissue microarray. ERBB2 FISH analysis used the Zytolight SPEC ERBB2/CEN 17 Dual Color Probe kit. Locus-specific ERBB2 probe (610-kb ZyGreen directly labeled fluorescent DNA probe) and CEP17 probe (ZyOrange, targeting sequences mapping in 17p11.1-q11.1 specific for the alpha satellite centromeric region D17Z1 of chromosome 17) were employed as per manufacturer's recommendations (ZytoVision GmbH, Bremerhaven, Germany). Nonoverlapping nuclei of tumor cells were assessed for HER2 probe and CEP17 probe signals. The FISH result was considered positive with a ratio HER2/CEP17  $\geq 2$  in  $\geq 50\%$  of tumor cells. Classification followed the HERACLES scoring system,<sup>17</sup> categorizing IHC staining into the following 4 groups: 0, 1+ (both "negative"), 2+ ("equivocal"), and 3+ ("positive"). ERBB2 amplification status was determined accordingly: IHC 0 or 1+ as "negative," IHC 2+ FISH negative as "negative," IHC 2+ FISH positive as "positive," and IHC 3+ as "positive."

**3' RNA sequencing.** In both cohorts, tumor RNA was extracted from macrodissected FFPE blocks from primary tumors using the Maxwell RSC RNA FFPE Kit (Promega). PolyA-RNA sequencing (RNA-seq) library preparation protocols were conducted using 400 ng of template RNA and the QuantSeq 3' mRNA-Seq Kit FWD for Illumina (Lexogen GmbH) following manufacturer's instructions. Libraries were sequenced on NovaSeq6000 (Illumina). FASTQ RNA-seq files were mapped using STAR aligner 2.7.9a, and raw read counts were obtained using the "Rsubread" package in R software (R Project for Statistical Computing). Before mapping, the genome index was built on GRCh38.p13 human genome. Samples with more than 10,000 genes detected were kept for further analysis. Batch correction was performed using the `Combat_seq` function from the "sva" package in R software. Mitochondrial and housekeeping genes were excluded from the raw count matrix. For each sample, the corresponding vector of raw counts was scaled: counts were divided by the total number of counts of the vector and multiplied by the median of the total number of counts across samples. Finally, the scaled data were log<sub>2</sub>-transformed.

**Multigene DNA sequencing.** Tumor DNA was extracted from macrodissected FFPE blocks from primary tumors. NGS was conducted with a dedicated panel of 92 amplicons (Ion AmpliSeq Colon-Lung Cancer Research Panel version 2; Life Technologies, Carlsbad, CA) covering >500 hotspot mutations in 22 genes, including *ERBB2*, *BRAF*, *KRAS*, *NRAS*, and *TP53*.

Multiplex polymerase chain reaction libraries were prepared using 30 ng of DNA whenever possible and 3  $\mu$ L of DNA for samples with DNA concentration <10 ng/ $\mu$ L by AmpliSeq technology (Ion AmpliSeq library kit, version 2, Ion library equalizer kit; Life Technologies). Clonal amplification and sequencing were done on the Ion Chef System (Ion PI Hi-Q Chef, Ion PI Chip Kit, version 3) and Ion Torrent Proton sequencer (Life Technologies). Data were analyzed by the Torrent Suite, versions 4.4.3 and 5.0.4 (Life Technologies) using optimized parameters: minimal depth of 300 $\times$  and detection thresholds of 2% and 1% for hotspots. Variant call files from the variant caller were loaded on a galaxy platform and annotated using the Safir2report tool.<sup>18</sup>

NGS, IHC, and FISH analyses for this study were performed and interpreted before 2015<sup>19</sup> and RNA-seq analysis were performed in 2019–2023.

## Statistical Analyses

Bioinformatic analyses were performed with R software, version 4.3.1.

ERBB2 NGS score was calculated to estimate ERBB2 copy number according to the following formula: (number of ERBB2 reads - number of ERBB2 reads expected per patient) / number of ERBB2 reads expected per patient. The number of ERBB2 reads expected per patient was determined by the total reads per patient multiplied by the mean ratio of ERBB2 reads to total reads in the series (minus extremes).

The optimal thresholds for "ERBB2 NGS score" and "ERBB2 RNA-seq expression" were determined using Youden's J-index method with the "cutpoint" package in R software. Differential gene expression analysis between IHC/FISH ERBB2-amplified and nonamplified ERBB2 samples was conducted based on RNA-seq data from PETACC-8 with the DESeq2 package. Nonhierarchical clustering with complete linkage method was performed on RNA-seq data from ERBB2 and 4 neighboring genes from PETACC8 and IDEA-France cohorts separately.

Associations between categorical variables were analyzed using Fisher exact test or  $\chi^2$  test as appropriate. Associations between categorical and continuous variables were assessed using Wilcoxon/Mann-Whitney test. Concordance between different diagnostic methods for ERBB2 amplification status was evaluated using Cohen's  $\kappa$  coefficient. Univariate Cox models analyzed time to recurrence (TTR) as a function of continuous ERBB2 RNA-seq expression, with a restricted cubic spline used for modeling. Univariate and multivariate Cox models were employed to analyze associations between clinicobiological variables and TTR. Kaplan–Meier method estimated TTR curves, which were compared using 2-sided log-rank test in the "survival" package in R software. Patients alive and relapse-free were censored at last follow-up and deceased patients without relapse were censored at date of death. Significant variables in univariate analyses ( $P < .05$ ) were included in multivariate analyses. Analyses were conducted with a 2-sided statistical significance level of 5%.

## Data Availability

All clinical and biological variables, as well as the RNA-seq counts used in this article, are available on request for academic researchers in the field of cancer research at the following e-mail addresses: [daniel.gonzalez@u-bourgogne.fr](mailto:daniel.gonzalez@u-bourgogne.fr) and [pierre.laurent-puig@parisdescartes.fr](mailto:pierre.laurent-puig@parisdescartes.fr).



## Results

### *ERBB2* Status in PETACC8 and IDEA-France Cohorts

**ERBB2 status in PETACC8 cohort.** Among PETACC8 patients, ERBB2 protein expression was evaluated using IHC in 1813 patients (88.7%) and the copy number of *ERBB2* gene was determined using FISH in 1691 patients (82.8%). According to the HERACLES criteria, we identified IHC/FISH *ERBB2* amplification in 34 cases (1.9%) and 1779 were considered as ERBB2 nonamplified.

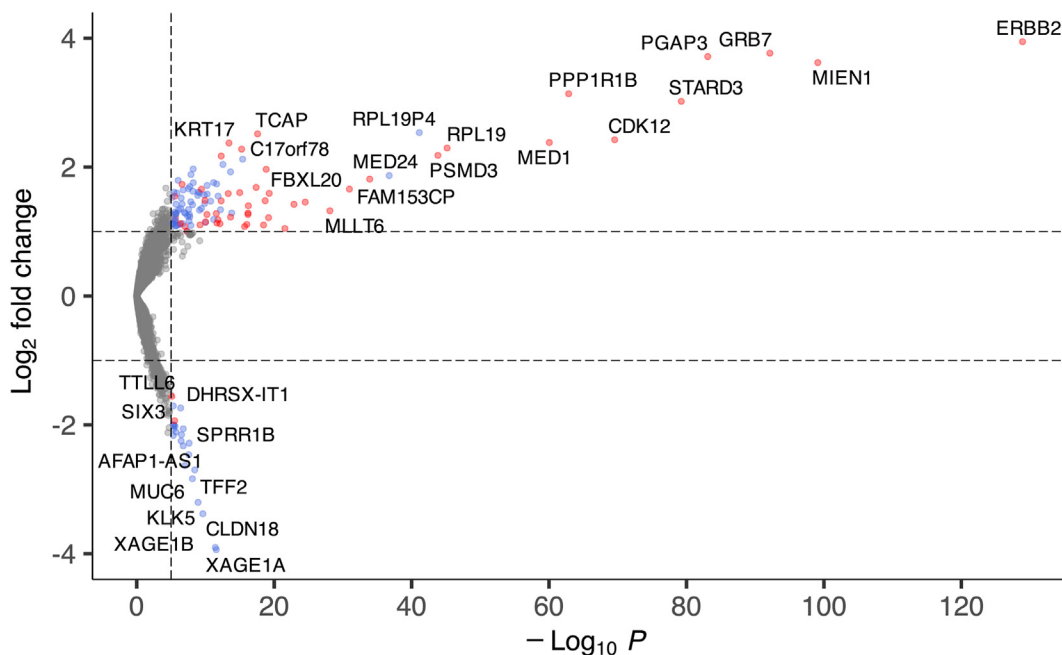
Using Youden's J-Index method, we calculated the optimal thresholds for *ERBB2* NGS score (n = 1719) and *ERBB2* RNA-seq expression (n = 1733) to maximize the sensitivity and specificity for identifying IHC/FISH *ERBB2*-amplified tumors. A high *ERBB2* NGS score (cutoff at 1.47) was observed in 33 patients (1.9%) and high *ERBB2* RNA-seq expression (cutoff at 9.34) was observed in 95 patients (5.5%). Somatic activating mutations of *ERBB2* were detected in 15 patients (0.9%) (Table 1 and Supplementary Table 1). Concordance rates between these diagnostic methods, measured by Cohen's  $\kappa$  coefficient, were 0.78 (95% CI, 0.66–0.90) for IHC/FISH vs NGS score and 0.40 (95% CI, 0.23–0.54) for IHC/FISH vs RNA-seq expression. Interestingly, we observed that high *ERBB2* RNA-seq expression was only mildly specific to amplified tumors, as some nonamplified samples also exhibited increased *ERBB2* RNA-seq expression. This suggests the existence of additional mechanisms besides amplification regulating *ERBB2* transcriptomic activity (Supplementary Figure 1).

**ERBB2 status in IDEA-France cohort.** Applying the same thresholds in the IDEA-France cohort allowed the identification of *ERBB2* NGS high score in 18 patients (1.6%) and *ERBB2* RNA-seq high expression in 55 patients (4.4%). In this cohort, somatic activating mutations of the *ERBB2* gene were found in 17 patients (1.5%) (Table 1).

**Identification of surrogate biomarkers of ERBB2 amplification.** We used RNA sequencing and differential gene expression analysis to compare IHC/FISH *ERBB2*-amplified and nonamplified tumors (Figure 1). As expected, the *ERBB2* gene exhibited the most pronounced increase in expression in IHC/FISH-amplified tumors compared with nonamplified tumors, showing the most significant *P* value. We observed that nearly all of the genes overexpressed in IHC/FISH-amplified tumors, compared with nonamplified tumors, were located on chromosome 17, specifically in the close vicinity of the *ERBB2* gene (17q12). Indeed, *ERBB2* gene amplification rarely occurs in isolation; most of the time, neighboring genes are also amplified, leading to their overexpression. Taking advantage of this co-amplification phenomenon, we investigated the expression of the following 4 *ERBB2* neighboring genes: *STARD3*, *PGAP3*, *MIEN1*, and *GRB7*. To establish a connection between regional amplification and RNA overexpression of the genes close to *ERBB2*, we performed nonhierarchical clustering and identified 2 clusters. In the PETACC8 cohort, cluster A included 28 of the 1733 tumors analyzed (1.6%), which exhibited overexpression of *ERBB2* neighboring genes (including *ERBB2* itself) (Figure 2). Among the 95 samples with high RNA-seq expression, 27 belonged to cluster A (28.4%). In contrast, 68 belonged to cluster B (71.6%), confirming that RNA-seq overexpression is only associated with regional amplification mechanisms in some cases. The concordance between cluster A tumors and the IHC/FISH *ERBB2*-amplified tumors in the PETACC8 cohort, as measured by Cohen's  $\kappa$  coefficient, was 0.74 (95% CI, 0.60–0.88). In the IDEA-France cohort, 15 patients (1.2%) belonged to cluster A. Among the 55 samples with high RNA-seq expression, 15 belonged to cluster A (27.3%) and 40 belonged to cluster B (72.7%). No patient with *ERBB2* NGS low score and *ERBB2* RNA-seq low expression belonged to cluster A (Supplementary Figure 2). To confirm

**Table 1.** *ERBB2* Status in PETACC8, IDEA-France, and Pooled Population

Characteristic	PETACC8 (N = 2043)	IDEA-France (N = 1612)	Pooled population (N = 3655)
<b><i>ERBB2</i> IHC/FISH</b>			
Positive, n (%)	34 (1.9)	—	34 (1.9)
Negative, n (%)	1779 (98.1)	—	1779 (98.1)
Undetermined, n	230	1612	1842
<b><i>ERBB2</i> NGS score</b>			
NGS high score, n (%)	33 (1.9)	18 (1.6)	51 (1.8)
NGS low score, n (%)	1686 (98.1)	1111 (98.4)	2797 (98.2)
Undetermined, n	324	483	807
<b><i>ERBB2</i> RNA expression</b>			
RNA high expression, n (%)	95 (5.5)	55 (4.4)	150 (5.0)
RNA low expression, n (%)	1638 (94.5)	1208 (95.6)	2846 (95.0)
Undetermined, n	310	349	659
<b><i>ERBB2</i> activating mutation</b>			
Mutated, n (%)	15 (0.9)	17 (1.5)	32 (1.1)
Nonmutated, n (%)	1681 (99.1)	1114 (98.5)	2795 (98.9)
Undetermined, n	347	481	828

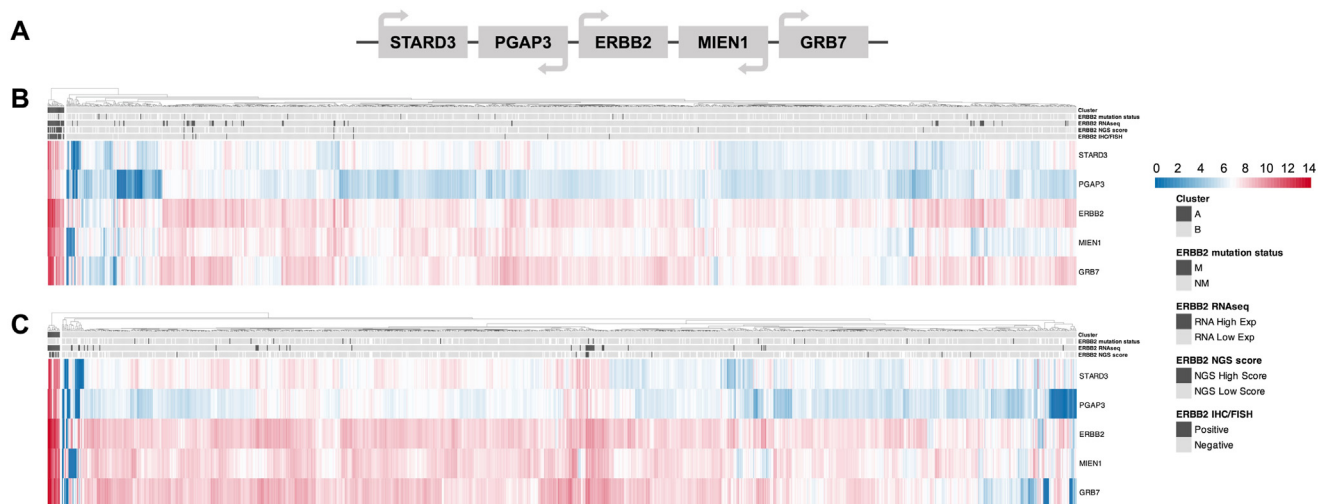


**Figure 1.** Volcano plot of IHC/FISH *ERBB2* differential gene expression analysis in PETACC8 cohort. Dashed lines correspond to  $P$  value cutoff of  $10^{-5}$  and  $\log_2$  fold-change of  $-1$  and  $1$  on  $x$ - and  $y$ -axis, respectively. Genes in red are located on chromosome 17.

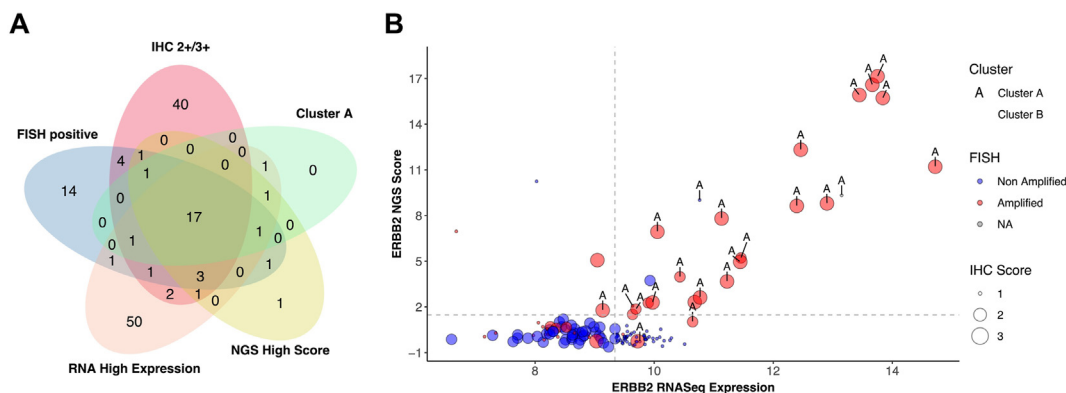
these results, we performed the same analysis on a cohort of 3409 breast cancers (SCAN-B cohort).<sup>16</sup> The concordance between cluster A tumors and the HER-amplified tumors in the SCAN-B cohort, as measured by Cohen's  $\kappa$  coefficient, was 0.785 (95% CI, 0.75–0.82) (Supplementary Figure 3).

**Comparative analysis between *ERBB2* diagnostic methods.** In the PETACC8 cohort, 1463 patients had complete data for all 5 methods (ie, IHC, FISH, RNA *ERBB2*

expression, NGS *ERBB2* score, and clustering) used to characterize *ERBB2* status. Of these, 141 tumor samples (9.6%) showed positivity in at least 1 of the techniques: 17 (12.1%) were concordant across all 5 techniques, 6 (4.3%) were concordant across 4 techniques, 4 (2.8%) were concordant across 3 techniques, 9 (6.4%) were concordant across 2 techniques, and 105 (74.5%) were positive with only 1 of the techniques. Among the latter group, 50 showed



**Figure 2.** Nonhierarchical clustering according to expression of *ERBB2* and 4 neighboring genes. (A) Schematic representation of neighboring genes at the *ERBB2* locus in the 17q12 region. (B, C) Heatmaps for PETACC8 (B) and IDEA-France (C) transcriptomic data. In the heatmap, each row represents a gene and each column represents a sample. The color and intensity of the boxes are used to represent changes in gene expression. Red represents up-regulated genes and blue represents down-regulated genes. Above the heatmap, the bars represent the distribution of samples according to *ERBB2* mutational status, *ERBB2* RNA-seq, *ERBB2* NGS score, and *ERBB2* status in IHC/FISH (only in PETACC8), respectively. The results of clustering are shown on top of each heatmap.



**Figure 3.** Comparative analysis between ERBB2 diagnostic methods in PETACC-8 cohort. (A) *Venn diagram* comparing the 5 diagnostic methods in PETACC8 (among the 141 patients with no missing data for IHC, FISH, ERBB2 NGS score, ERBB2 RNA-seq expression and clustering, and showing positivity in at least 1 of the techniques). (B) ERBB2 RNA-seq expression as a function of ERBB2 NGS score according to FISH status, IHC score, and cluster membership in the 145 samples from PETACC8, with at least 1 positive test and no missing data for RNA-seq and NGS tests. Samples without a label belong to cluster B. Dashed lines correspond to thresholds for ERBB2 RNA-seq expression and ERBB2 NGS score on x- and y-axis, respectively.

only ERBB2 RNA-seq high expression, and 40 showed only IHC 2+ staining (Figure 3A). Similar to previous studies,<sup>17</sup> all cases (19 of 19) with IHC 3+ showed amplification by FISH, and only a minority of patients (9 of 52 [17%]) with equivocal IHC (2+) were FISH-positive. Conversely, 17 patients classified as amplified by FISH tested negative on IHC, indicating likely partial amplification of the ERBB2 gene or a false-positive FISH result.

**Toward a new molecular definition of ERBB2 amplification by the combination of RNA and DNA testing.** Based on the previous results, we defined a new molecular classification of ERBB2 status based on a combination of RNA and DNA testing and refer to it as “molecular ERBB2-amplified” in the following paragraphs. Samples belonging to this new molecular ERBB2-amplified group exhibit either RNA overexpression of ERBB2 and its neighboring genes (cluster A) or high ERBB2 NGS score and high RNA-seq expression of ERBB2 (eg, samples with ERBB2 focal amplification). In the PETACC8 cohort, the concordance measured by Cohen’s  $\kappa$  coefficient between molecular ERBB2 amplification and IHC/FISH test is 0.81 (95% CI, 0.70–0.92). The prevalence of molecular ERBB2-amplified status is 1.85% in the PETACC8 cohort (n = 32 of 1726) and 1.5% in the IDEA cohort (n = 19 of 1253).

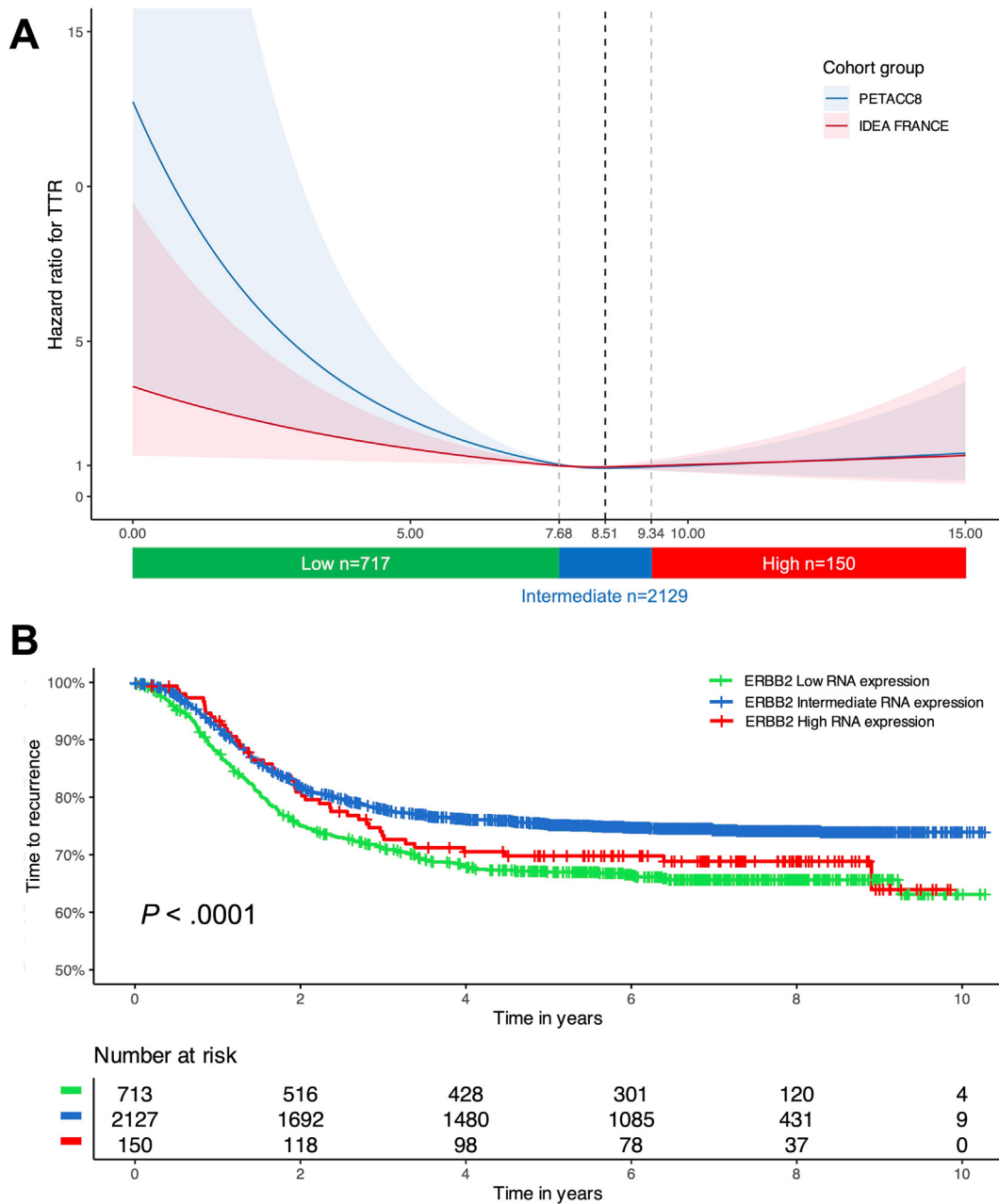
Considering the 3 potential mechanisms of ERBB2 pathway activation, that is, the amplification of the ERBB2 gene or the presence of an activating mutation in the catalytic domain of ERBB2, we combined the molecular ERBB2 status with the ERBB2 mutational status. In both cohorts, 81 patients belonged to the “molecular ERBB2-amplified or ERBB2 mutated” group.

**Clinicobiological characteristics associated with ERBB2 amplification or ERBB2 mutation in PETACC8 cohort.** Tumors with ERBB2 amplification determined by IHC/FISH were more frequently located in the distal part of the colon (73.5% vs 60.1%;  $P = .014$ ), were more often TP53-mutated (83.3% vs 60.4%;  $P = .011$ ), and none of them were deficient mismatch repair (0% vs 10.1%;  $P = .043$ ). A similar trend was observed in molecular ERBB2-amplified tumors, which were more frequently located

distally (78.1% vs 60.0%;  $P = .002$ ), had an even greater prevalence of TP53 mutations (92.6% vs 59.9%;  $P < .001$ ), and were all proficient mismatch repair (0% vs 10.2%;  $P = .068$ ). In addition, they were less frequently RAS-mutated (22.2% vs 49.0%;  $P = .006$ ) (Supplementary Table 2). Tumors with ERBB2 somatic activating mutations were more frequently deficient mismatch repair than nonmutated ERBB2 tumors (35.7% vs 10.3%;  $P = .011$ ). They were more commonly classified in the consensus molecular subtype (CMS) as CMS1 (57.1% vs 20.6%;  $P = .009$ ).<sup>20</sup> There was no significant difference between mutated and nonmutated tumors in the remaining tested clinical and molecular variables (Supplementary Table 2). In addition, there was no association between ERBB2 RNA-seq expression and ERBB2-activating mutations ( $P = .36$ ) (Supplementary Figure 4).

### Survival Analysis According to ERBB2 Status in PETACC8 and IDEA-France Pooled Cohorts

Although several studies have been conducted during the last decade, the prognostic role of ERBB2 alterations in CC continues to be debated. To better understand the complex role of ERBB2 in the prognosis of stage III CC, we investigated survival based on ERBB2 transcriptomic expression level as a continuous variable. Interestingly, ERBB2 RNA expression and TTR were significantly associated in the univariate Cox model in the PETACC8 cohort (hazard ratio [HR], 0.88; 95% CI, 0.79–0.98;  $P = .021$ ), indicating a protective effect of ERBB2 expression level on TTR. However, previous reports suggested a pejorative prognostic value of ERBB2 amplification.<sup>5</sup> Therefore, we performed a restricted cubic spline regression, revealing a nonlinear relationship between ERBB2 RNA-seq expression level and survival in PETACC8 and IDEA-France cohorts (Figure 4A). Interestingly, our findings suggested that patients with either low or high ERBB2 transcript levels in their tumors have a shorter TTR than those with intermediate levels. This pattern was consistent across both cohorts. We observed a very similar trend in the SCAN-B cohort of breast cancers, thus confirming the results identified in our



**Figure 4.** Survival analysis according to ERBB2 expression status. (A) *Restricted cubic spline curves* of the risk of relapse according to ERBB2 RNA-seq expression value in PETACC8 and IDEA-France cohorts. (B) *Kaplan-Meier TTR curves* according to ERBB2 RNA-seq group expression in PETACC8 and IDEA-France pooled population.

2 CC cohorts and extending our findings to other ERBB2-dependent tumor types (Supplementary Figure 5).

We divided the PETACC8 and IDEA-France pooled population into 3 categories based on ERBB2 RNA-seq expression levels, centered around a pivotal value of 8.51, correlating with the lowest HR for tumor recurrence. These groups were categorized as follows: "low expression" with ERBB2 RNA-seq levels  $<7.68$  ( $n = 717$ ), "intermediate expression" ranged from 7.68 to 9.34 ( $n = 2129$ , reference group), and "high expression" was  $\geq 9.34$  ( $n = 150$ ), which was the previously set threshold. Results of Kaplan-Meier analysis showed a significant difference in TTR among these groups ( $P < .0001$ ). Patients with low ERBB2

expression had the poorest prognosis, with a 3-year TTR of 71.2%, compared with 73.3% and 77.9% for those with high and intermediate ERBB2 expression, respectively (Figure 4B and Supplementary Figure 6). Compared with the intermediate ERBB2 expression group, tumors with low ERBB2 expression were more often located in the proximal colon (45.9% vs 37.4%;  $P < .001$ ), had higher rates of RAS mutations (53.3% vs 48.3%;  $P = .028$ ) and BRAF mutations (15.9% vs 10.1%;  $P < .001$ ), were more frequently classified as pT4 stage (24.3% vs 19.5%;  $P = .007$ ), had higher histopathology grading (19.4% vs 12.5%;  $P < .001$ ), and were more likely to fall into CMS1 and CMS4 categories (20.5% vs 15.8% and 40.9% vs 23.0%, respectively;  $P < .001$ )



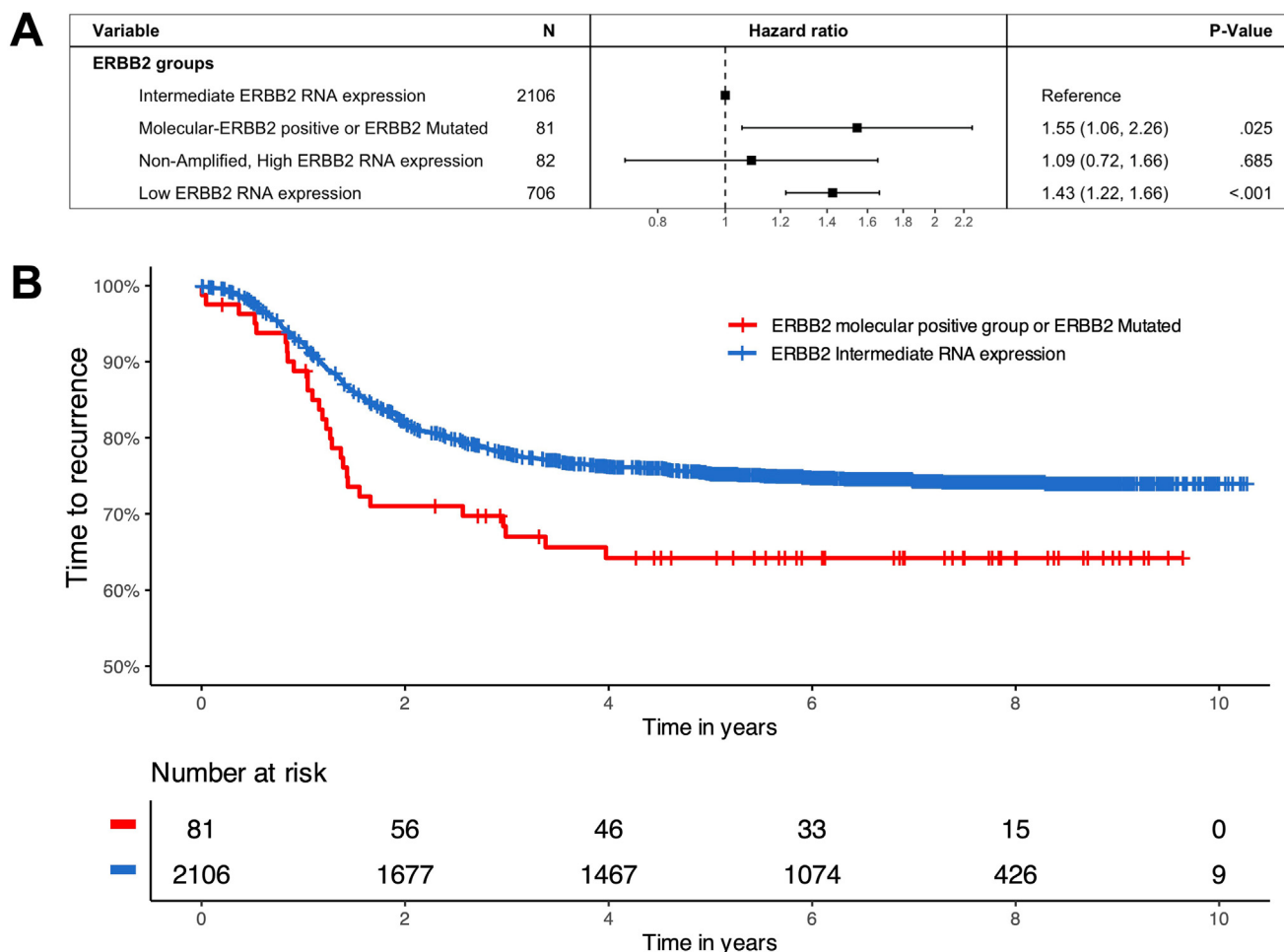
(Supplementary Table 3). Low *ERBB2* expression was prognostic in patients with CMS2 and CMS4 (HR, 1.45; 95% CI, 1.08–1.94;  $P = .013$ ; HR, 1.31; 95% CI, 1.03–1.68;  $P = .028$ ) (Supplementary Figure 7). Among patients with low *ERBB2* expression tumors, those treated with FOLFOX plus cetuximab had shorter TTR than those receiving FOLFOX alone (Supplementary Figure 8A). This observation persisted across *RAS* wild-type or *RAS*-mutated tumors (Supplementary Figure 9). Conversely, in high or intermediate *ERBB2* expression groups, no significant outcome difference was observed between treatment arms (Supplementary Figure 8B). When treated with FOLFOX alone, lower (quartile 1) and higher (quartile 4) *ERBB2* expression quartile groups have similar TTR; however, when treated with FOLFOX + cetuximab, the 2 groups displayed opposite responses (Supplementary Figure 8C).

We found that high *ERBB2* RNA expression without amplification did not have prognostic significance in the overall population (Figure 5A). Conversely, TTR was significantly shorter for patients activating ERBB2 either by harboring molecular ERBB2-amplified status or ERBB2 mutations, compared with the group with intermediate *ERBB2* expression (HR, 1.5; 95% CI, 1.06–2.3;  $P = .025$ ; 3-

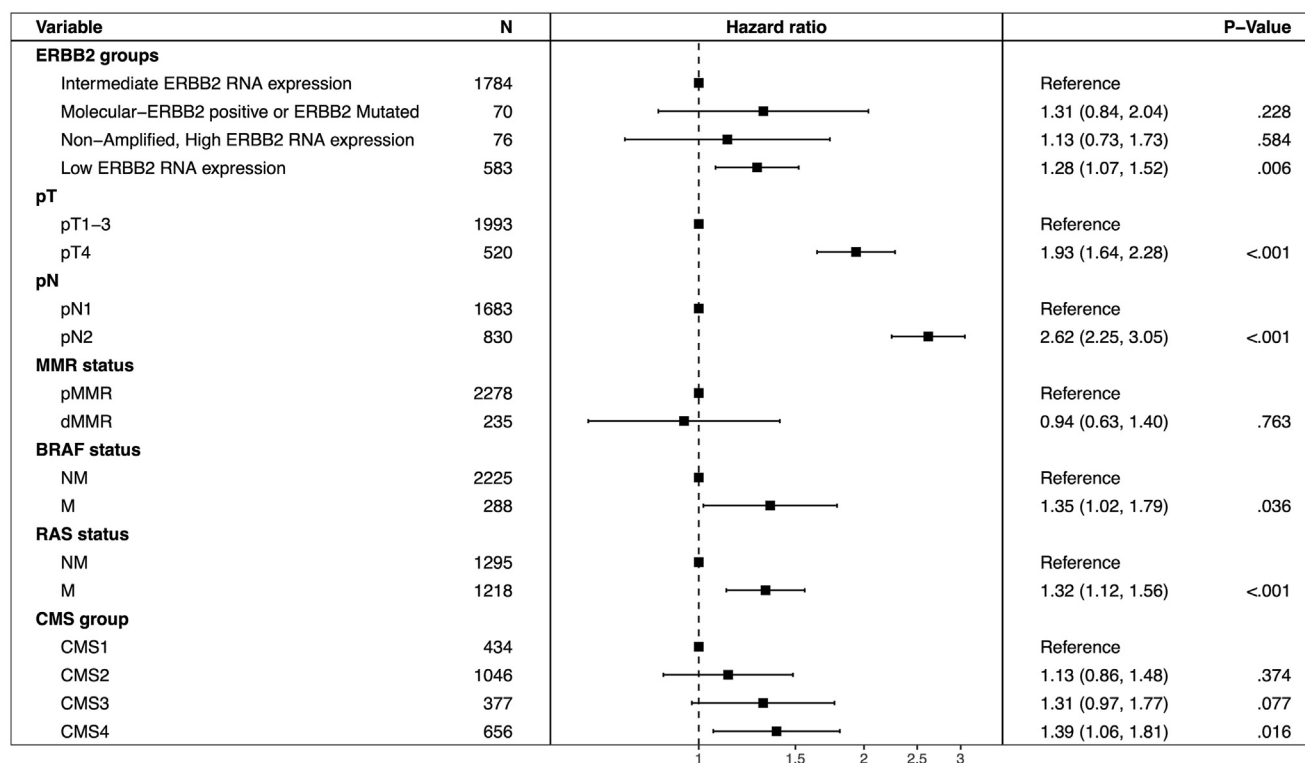
year TTR: 67.0% vs 78.0%) (Figure 5B). In the multivariate analysis using a Cox proportional hazard model, the low *ERBB2* expression group was confirmed as a significant independent factor of bad prognosis (HR, 1.28; 95% CI, 1.07–1.52;  $P = .006$ ) (Figure 6). Conversely, the molecular ERBB2-amplified or ERBB2-mutated group was not more significant when adjusting for the other prognostic factors in the multivariate model.

## Discussion

Selecting patients with cancer with rare genetic events is difficult and costly and raises the question of a parsimonious use of tissues. Furthermore, the development of new technologies allowing the characterization of numerous genetic alterations, leading to the simultaneous detection of multiple markers, raises the question of developing alternative strategies to the classical detection of theragnostic markers. Assessing ERBB2 amplification status in CC traditionally involves IHC followed by FISH when ambiguity arises. Although these techniques have long served as the gold standard in clinical practice, they are difficult to standardize and may lead to false-negative results.<sup>21,22</sup> For



**Figure 5.** Survival analysis according to ERBB2 expression and mutational status. (A) Forest plot for TTR according to ERBB2 status in PETACC8 and IDEA-France pooled population. (B) Kaplan–Meier curves for TTR according to ERBB2 genomic status (amplification or mutation) in PETACC8 and IDEA-France pooled population.



**Figure 6.** Forest plot based on the results of multivariate analysis of the factors associated with TTR in PETACC8 and IDEA-France pooled population.

example, the pre-analytical phase (eg, time and type of fixative and old blank slides) may influence IHC sensitivity and, to a lesser extent, FISH. To mitigate these risks, we ensured that IHC and FISH were performed on fresh sections, cut within 24 hours of testing, to maintain optimal quality. Nevertheless, traditional methods are being challenged by the advent of new DNA and RNA sequencing technologies, which have emerged as powerful tools for characterizing CC's molecular landscape, simultaneously analyzing various molecular features in a standardized and efficient quantitative approach.

In our study, NGS and RNA-seq revealed ERBB2 amplification in approximately 1.7% of CC cases, which aligns with previous studies using IHC/FISH.<sup>4</sup> Although the NGS score demonstrated excellent concordance with IHC/FISH in our study and multiple other studies,<sup>23-25</sup> 7 patients (0.43%) showed an ERBB2 amplification by NGS without positive IHC/FISH. Data from the MOUNTAINEER trial<sup>26</sup> demonstrated that among 10 patients negative by central HER2 IHC/FISH, the only patient who responded to HER2-targeted therapy was found to be ERBB2-amplified by NGS. This suggests that NGS may identify additional patients who could benefit from targeted therapy, even when traditional IHC/FISH methods indicate a negative result. However, ERBB2 RNA and protein overexpression, identified by RNA-seq and IHC without DNA amplification, may be attributed to transcriptional regulations, such as the activation of transcription factors influencing ERBB2 expression. In that subgroup of patients, sensitivity to HER2 inhibitors remains to be explored in both digestive and breast cancers.

To explore these variations, we expanded our analysis beyond ERBB2 RNA expression, examining the RNA expression of neighboring genes that may be co-amplified, but are not transcriptionally co-regulated. This broader approach allows for extrapolating ERBB2 amplification status from whole transcriptome data, applicable to patients analyzed via RNA-seq or transcriptome microarrays. Furthermore, this method can potentially identify other amplified genes in CC, such as KRAS, EGFR, or MET.

Currently, anti-HER2 treatments are being developed in randomized trials for ERBB2-amplified, RAS wild-type metastatic CC, with promising efficacy and acceptable tolerability reported in phase 2 trials using compounds such as tucatinib and trastuzumab-deruxtecan. Given the tumor shrinkage observed with these new drugs, they are now being tested in neoadjuvant proof-of-concept platforms, such as the NEO French platform in collaboration with the FOxTROT UK platform (NEO-HER2 trial) and the Unicorn Italian platform. Patients prioritized for screening would likely be those with high-risk stage III disease who could benefit from an intensified preoperative treatment approach. In this context, having comprehensive data from a large patient population on the outcomes of different HER2 profiles in stage III CC, before the introduction of anti-HER2 therapies, will be essential for developing accurate statistical models and hypotheses for future clinical trials evaluating these treatments.

Regarding prognostic impact, the low incidence of this alteration hampers the assessment of the potential prognostic effect of ERBB2 amplification in CC. Up-to-date studies present divergent results, although there is a trend toward an association between ERBB2 amplification and

less favorable overall or progression-free survival. We showed here that these inconsistencies could also be explained by the fact that, in addition to *ERBB2* overexpression, tumors with low *ERBB2* expression are also associated with a guarded prognosis. Furthermore, we demonstrated that patients with low *ERBB2*-expressing tumors are more frequently located in the proximal colon and, when treated with FOLFOX plus cetuximab—an EGFR-blocking antibody—have poorer survival than patients treated with FOLFOX alone. Together, these results could partly explain the lower impact of cetuximab treatment in proximal CC compared with distal CC.<sup>27</sup> Although HER2 amplification has been suggested previously as a negative predictor of response to cetuximab,<sup>28</sup> our findings suggested that this may not hold true for RNA expression (Supplementary Figure 8C). Indeed, patients with high *ERBB2* RNA expression appear to derive benefits from anti-EGFR treatment. Similar results were reported recently by Battaglin et al<sup>29</sup> in a cohort of metastatic colorectal cancer with a nonlinear association between *ERBB2* expression and outcome, particularly in association with cetuximab treatment. By blocking EGFR activation, cetuximab may prevent the formation of heterodimers with *ERBB2*. This action could be particularly beneficial in patients with high *ERBB2* expression, as it might negatively modulate the mitogen-activated protein kinase signaling pathway, thereby reducing cell proliferation. Overall, these findings suggest that although patients with HER2 amplification may benefit from targeted anti-HER2 therapies, those with elevated *ERBB2* RNA expression could potentially respond favorably to cetuximab, underscoring the theragnostic value of molecular profiling in optimizing treatment strategies for colorectal cancer.

The results presented in this study provide valuable insights into the prevalence and prognostic significance of *ERBB2* amplification, molecular alterations, and expression in CC. Our study suggests a Janus aspect of the expression of *ERBB2*. The nonlinear relation between the *ERBB2* expression and TTR was evidenced for the first time in stage III CC and breast cancer.

These findings refine the prognostic implications of such alterations, introducing the potential role of assessing *ERBB2* status in stage III colorectal cancer. Despite *ERBB2* amplification being a relatively rare event, the considerable efficacy and tolerable safety profile demonstrated by anti-*ERBB2* drugs in patients with chemorefractory disease underscore the importance of early *ERBB2* screening to assist clinicians in selecting optimal treatment options and directing patients toward relevant clinical trials.

## Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at [www.gastrojournal.org](http://www.gastrojournal.org), and at <http://doi.org/10.1053/j.gastro.2024.10.046>.

## References

1. Taieb J, Gallois C. Adjuvant chemotherapy for stage III colon cancer. *Cancers* 2020;12:2679.
2. Argilés G, Tabernero J, Labianca R, et al. Localised colon cancer: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. *Ann Oncol* 2020; 31:1291–1305.
3. De Cuyper A, Van Den Eynde M, Machiels J-P. HER2 as a predictive biomarker and treatment target in colorectal cancer. *Clin Colorectal Cancer* 2020;19:65–72.
4. Sartore-Bianchi A, Trusolino L, Martino C, et al. Dual-targeted therapy with trastuzumab and lapatinib in treatment-refractory, KRAS codon 12/13 wild-type, HER2-positive metastatic colorectal cancer (HERACLES): a proof-of-concept, multicentre, open-label, phase 2 trial. *Lancet Oncol* 2016;17:738–746.
5. Siena S, Sartore-Bianchi A, Marsoni S, et al. Targeting the human epidermal growth factor receptor 2 (HER2) oncogene in colorectal cancer. *Ann Oncol* 2018; 29:1108–1119.
6. Ahcene Djballah S, Daniel F, Milani A, et al. HER2 in colorectal cancer: the long and winding road from negative predictive factor to positive actionable target. *Am Soc Clin Oncol Educ Book* 2022;42:1–14.
7. Guarini C, Grassi T, Pezzicoli G, et al. Beyond RAS and BRAF: HER2, a new actionable oncotarget in advanced colorectal cancer. *Int J Mol Sci* 2021;22:6813.
8. Meric-Bernstam F, Hurwitz H, Raghav KPS, et al. Pertuzumab plus trastuzumab for HER2-amplified metastatic colorectal cancer (MyPathway): an updated report from a multicentre, open-label, phase 2a, multiple basket study. *Lancet Oncol* 2019;20:518–530.
9. Sartore-Bianchi A, Lonardi S, Martino C, et al. Pertuzumab and trastuzumab emtansine in patients with HER2-amplified metastatic colorectal cancer: the phase II HERACLES-B trial. *ESMO Open* 2020;5:e000911.
10. Siena S, Di Bartolomeo M, Raghav K, et al. Trastuzumab deruxtecan (DS-8201) in patients with HER2-expressing metastatic colorectal cancer (DESTINY-CRC01): a multicentre, open-label, phase 2 trial. *Lancet Oncol* 2021; 22:779–789.
11. Strickler JH, Cercek A, Siena S, et al. Tucatinib plus trastuzumab for chemotherapy-refractory, HER2-positive, RAS wild-type unresectable or metastatic colorectal cancer (MOUNTAINEER): a multicentre, open-label, phase 2 study. *Lancet Oncol* 2023;24:496–508.
12. Ross JS, Fakhri M, Ali SM, et al. Targeting HER2 in colorectal cancer: the landscape of amplification and short variant mutations in *ERBB2* and *ERBB3*. *Cancer* 2018;124:1358–1373.
13. Yoshino T, Di Bartolomeo M, Raghav K, et al. Final results of DESTINY-CRC01 investigating trastuzumab deruxtecan in patients with HER2-expressing metastatic colorectal cancer. *Nat Commun* 2023;14:3332.
14. Taieb J, Tabernero J, Mini E, et al. Oxaliplatin, fluorouracil, and leucovorin with or without cetuximab in patients with resected stage III colon cancer (PETACC-8): an open-label, randomised phase 3 trial. *Lancet Oncol* 2014;15:862–873.
15. André T, Vernerey D, Mineur L, et al. Three versus 6 months of oxaliplatin-based adjuvant chemotherapy for patients with stage III colon cancer: disease-free survival results from a randomized, open-label, international

- Duration Evaluation of Adjuvant (IDEA) France, phase III trial. *J Clin Oncol* 2018;36:1469–1477.
16. Brueffer C, Vallon-Christersson J, Grabau D, et al. Clinical value of RNA sequencing-based classifiers for prediction of the five conventional breast cancer biomarkers: a report from the population-based multi-center Sweden Cancerome Analysis Network-Breast Initiative. *JCO Precis Oncol* 2018;2:PO.17.00135.
  17. Valtorta E, Martino C, Sartore-Bianchi A, et al. Assessment of a HER2 scoring system for colorectal cancer: results from a validation study. *Mod Pathol* 2015; 28:1481–1491.
  18. Digan W, Countouris H, Barritault M, et al. An architecture for genomics analysis in a clinical setting using Galaxy and Docker. *GigaScience* 2017;6:gix099.
  19. Laurent-Puig P, Balogoun R, Cayre A, et al. ERBB2 alterations a new prognostic biomarker in stage III colon cancer from a FOLFOX based adjuvant trial (PETACC8). *Ann Oncol* 2016;27:vi151.
  20. Marisa L, Blum Y, Taieb J, et al. Intratumor CMS heterogeneity impacts patient prognosis in localized colon cancer. *Clin Cancer Res* 2021;27:4768–4780.
  21. Khoury T, Sait S, Hwang H, et al. Delay to formalin fixation effect on breast biomarkers. *Mod Pathol* 2009; 22:1457–1467.
  22. Wolff AC, Hammond MEH, Hicks DG, et al. Recommendations for human epidermal growth factor receptor 2 testing in breast cancer: American Society of Clinical Oncology/College of American Pathologists Clinical Practice Guideline Update. *J Clin Oncol* 2013;31:3997–4013.
  23. Cenaj O, Ligon AH, Hornick JL, et al. Detection of ERBB2 amplification by next-generation sequencing predicts HER2 expression in colorectal carcinoma. *Am J Clin Pathol* 2019;152:97–108.
  24. Edenfield WJ, Chung KY, Gatalica Z, et al. Molecular profiling of HER2-positive colorectal cancer for identification of multiple potential drug targets. *J Clin Oncol* 2014;32:e14508–e14508.
  25. Fujii S, Magliocco AM, Kim J, et al. International harmonization of provisional diagnostic criteria for ERBB2-amplified metastatic colorectal cancer allowing for screening by next-generation sequencing panel. *JCO Precision Oncology* 2020;4. <https://doi.org/10.1200/PO.19.00154>.
  26. Strickler JH, Cercek A, Ng K, et al. HER2 testing in the MOUNTAINEER trial: analysis of treatment response based on central HER2 assessment using IHC/ISH and NGS. *J Clin Oncol* 2023;41:3528–3528.
  27. Brulé SY, Jonker DJ, Karapetis CS, et al. Location of colon cancer (right-sided versus left-sided) as a prognostic factor and a predictor of benefit from cetuximab in NCIC CO.17. *Eur J Cancer* 2015;51:1405–1414.
  28. Sartore-Bianchi A, Amatu A, Porcu L, et al. HER2 positivity predicts unresponsiveness to EGFR-targeted treatment in metastatic colorectal cancer. *Oncologist* 2019;24:1395–1402.
  29. Battaglin F, Ou F-S, Qu X, et al. HER2 gene expression levels are predictive and prognostic in patients with metastatic colorectal cancer enrolled in CALGB/SWOG 80405. *J Clin Oncol* 2024;JCO.23.01507.

---

Received July 12, 2024. Accepted October 22, 2024.

#### Correspondence

Address correspondence to: Camilla Pilati, PhD, Centre de Recherche des Cordeliers, 15 rue de l'école de médecine, Paris 75006, France. e-mail: [camilla.pilati@inserm.fr](mailto:camilla.pilati@inserm.fr).

#### Acknowledgments

Camilla Pilati and Audrey Soulabaille contributed equally to this work.

#### Credit Authorship Contributions

Camilla Pilati, PhD (Conceptualization: Lead; Formal analysis: Lead; Methodology: Equal; Project administration: Equal; Supervision: Equal; Validation: Equal; Visualization: Lead; Writing – original draft: Lead)

Audrey Soulabaille, MD (Conceptualization: Equal; Formal analysis: Lead; Methodology: Equal; Visualization: Equal; Writing – original draft: Equal)

Claire Gallois, MD, PhD (Data curation: Equal; Formal analysis: Supporting; Resources: Equal)

Hélène Blons, PharmD, PhD (Data curation: Supporting; Resources: Equal)

Anne Cayre, PhD (Investigation: Equal; Resources: Supporting)

Marine Sroussi, MD (Data curation: Supporting; Formal analysis: Equal)

Delphine Le Corre, MS (Data curation: Supporting; Investigation: Equal)

Sophie Mouillet-Richard, PhD (Conceptualization: Equal; Funding acquisition: Supporting; Supervision: Supporting)

Claire Mulet, MS (Data curation: Supporting; Investigation: Supporting)

Karine Le Malicot, PhD (Data curation: Supporting; Formal analysis: Supporting)

Aurélien De Reynies, PhD (Formal analysis: Supporting)

Jean-Baptiste Bachet, MD, PhD (Resources: Equal)

Christophe Borg, MD, PhD (Resources: Equal)

Frédéric Di Fiore, MD, PhD (Resources: Equal)

Rosine Guimbaud, MD, PhD (Resources: Equal)

Jaafar Bennouna, MD, PhD (Resources: Equal)

Thierry André, MD, PhD (Resources: Equal)

Julien Taieb, MD, PhD (Resources: Lead)

Frédérique Penault-Llorca, MD, PhD (Data curation: Supporting; Formal analysis: Equal; Investigation: Equal; Resources: Supporting)

Pierre Laurent-Puig, MD, PhD (Conceptualization: Lead; Formal analysis: Equal; Funding acquisition: Lead; Methodology: Equal; Project administration: Equal; Supervision: Lead; Validation: Equal; Writing – original draft: Lead)

#### Conflicts of interest

The authors disclose no conflicts.

#### Funding

This work was supported by Aide et Recherche en Cancérologie Digestive Fondation and Association pour la Recherche sur le Cancer.



**Supplementary Table 1.** *ERBB2*-Activating Mutations Observed in PETACC8 and IDEA-France Cohorts

Variable	PETACC8 (n = 15)		IDEA-France (n = 19 <sup>a</sup> )	
	n	Case with amplification	n	Case with amplification
Mutation type				
Missense				
p.Leu755Ser	3	1 <sup>b</sup>	8	1 <sup>c</sup>
p.Val842Ile	5	—	3	—
p.Val777Leu	3	—	1	—
p.Asp769Tyr	1	—	2	—
p.Val859Ile	1	—	—	—
p.Gly882Ala	1	—	—	—
p.Ala771Val	—	—	1	—
p.Asn857His	—	—	1	—
p.Asp873Gly	—	—	1	—
p.Leu755Pro	—	—	1	—
Deletion	—	—	—	—
p.Glu770_splice	1	—	—	—
Insertion/deletion	—	—	—	—
p.Ala775_Gly776insValAla	—	—	1	—

<sup>a</sup>19 mutations in 17 tumor samples (2 patients with *ERBB2* double mutation).

<sup>b</sup>*ERBB2* status: IHC/FISH-positive, NGS high score, RNA-seq high expression, molecular *ERBB2*-amplified.

<sup>c</sup>*ERBB2* status: NGS high score, RNA-seq high expression, molecular *ERBB2*-amplified.

**Supplementary Table 2.** Clinicobiological Characteristics Associated With Immunohistochemistry and Fluorescence in Situ Hybridization Status, Molecular ERBB2 Status, and ERBB2 Mutation Status in PETACC8 Cohort

Characteristic	IHC/FISH status			Molecular ERBB2 status			ERBB2 mutation status		
	Positive (n = 34)	Negative (n = 1779)	<i>P</i> value <sup>a</sup>	Amplified (n = 32)	Negative (n = 1694)	<i>P</i> value <sup>a</sup>	M (n = 15)	NM (n = 1681)	<i>P</i> value <sup>a</sup>
Age, n (%)			.3			.3			>.9
<70 y	32 (94.1)	1542 (86.7)		30 (93.8)	1461 (86.2)		13 (86.7)	1453 (86.4)	
≥70 y	2 (5.9)	237 (13.3)		2 (6.3)	233 (13.8)		2 (13.3)	228 (13.6)	
Gender, n (%)			.2			.037			.2
Female	11 (32.4)	764 (42.9)		8 (25.0)	735 (43.4)		9 (60.0)	725 (43.1)	
Male	23 (67.6)	1015 (57.1)		24 (75.0)	959 (56.6)		6 (40.0)	956 (56.9)	
WHO			.034			.3			.2
0, n (%)	23 (67.6)	1403 (81.9)		23 (74.2)	1335 (81.8)		10 (66.7)	1320 (81.4)	
≥1, n (%)	11 (32.4)	311 (18.1)		8 (25.8)	298 (18.2)		5 (33.3)	301 (18.6)	
Unknown, n	0	65		1	61		0	60	
Tumor site			.014			.002			.3
Proximal, n (%)	7 (20.6)	687 (38.8)		5 (15.6)	656 (38.9)		9 (60.0)	656 (39.2)	
Distal, n (%)	25 (73.5)	1065 (60.1)		25 (78.1)	1012 (60.0)		6 (40.0)	999 (59.7)	
Dual, n (%)	2 (5.9)	19 (1.1)		2 (6.3)	18 (1.1)		0 (0.0)	19 (1.1)	
Unknown, n	0	8		0	8		0	7	
Bowel obstruction and/or perforation, n (%)			.3			.085			>.9
Yes	9 (26.5)	336 (18.9)		10 (31.3)	324 (19.1)		3 (20.0)	315 (18.7)	
No	25 (73.5)	1443 (81.1)		22 (68.8)	1370 (80.9)		12 (80.0)	1366 (81.3)	
pT			.10			.066			>.9
pT1–3, n (%)	23 (67.6)	1407 (79.1)		21 (65.6)	1338 (79.0)		12 (80.0)	1328 (79.0)	
pT4, n (%)	11 (32.4)	371 (20.9)		11 (34.4)	355 (21.0)		3 (20.0)	353 (21.0)	
Unknown, n	0	1		0	1				
pN			.3			.4			.2
pN1, n (%)	18 (52.9)	1110 (62.4)		18 (56.3)	1064 (62.8)		7 (46.7)	1048 (62.3)	
pN2, n (%)	16 (47.1)	669 (37.6)		14 (43.8)	630 (37.2)		8 (53.3)	633 (37.7)	
Histopathology grading			.5			.2			.7
G1–2, n (%)	26 (76.5)	1428 (81.3)		23 (71.9)	1362 (81.4)		11 (78.6)	1337 (80.6)	
G3–4, n (%)	8 (23.5)	328 (18.7)		9 (28.1)	312 (18.6)		3 (21.4)	322 (19.4)	
Unknown, n	0	23		0	20		1	22	
RAS			.10			.006			.6
M, n (%)	10 (33.3)	797 (48.6)		6 (22.2)	767 (49.0)		8 (53.3)	774 (46.0)	
NM, n (%)	20 (66.7)	843 (51.4)		21 (77.8)	799 (51.0)		7 (46.7)	907 (54.0)	
Unknown, n	4	139		5	128		0	0	

Supplementary Table 2. Continued

Characteristic	IHC/FISH status			Molecular ERBB2 status			ERBB2 mutation status		
	Positive (n = 34)	Negative (n = 1779)	<i>P</i> value <sup>a</sup>	Amplified (n = 32)	Negative (n = 1694)	<i>P</i> value <sup>a</sup>	M (n = 15)	NM (n = 1681)	<i>P</i> value <sup>a</sup>
<b>BRAF</b>			.2			.2			>.9
M, n (%)	1 (2.9)	195 (11.5)		1 (3.1)	183 (11.3)		1 (6.7)	197 (11.7)	
NM, n (%)	33 (97.1)	1504 (88.5)		31 (96.9)	1438 (88.7)		14 (93.3)	1484 (88.3)	
Unknown, n	0	80		0	73		0	0	
<b>MMR</b>			.043			.068			.011
MSI/dMMR, n (%)	0 (0.0)	177 (10.1)		0 (0.0)	170 (10.2)		5 (35.7)	161 (10.3)	
MSS/pMMR, n (%)	34 (100.0)	1580 (89.9)		32 (100.0)	1491 (89.8)		9 (64.3)	1403 (89.7)	
Unknown, n	0	22		0	33		1	117	
<b>CMS</b>			.3			.3			.009
CMS1, n (%)	3 (9.4)	339 (20.3)		4 (12.5)	340 (20.2)		8 (57.1)	309 (20.6)	
CMS2, n (%)	20 (62.5)	764 (45.8)		19 (59.4)	767 (45.7)		3 (21.4)	689 (46.0)	
CMS3, n (%)	2 (6.3)	187 (11.2)		1 (3.1)	192 (11.4)		2 (14.3)	162 (10.8)	
CMS4, n (%)	7 (21.9)	379 (22.7)		8 (25.0)	381 (22.7)		1 (7.1)	338 (22.6)	
Unknown, n	2	110		0	14		1	183	
<b>TP53</b>			.011			<.001			.3
M, n (%)	25 (83.3)	952 (60.4)		25 (92.6)	903 (59.9)		7 (46.7)	1023 (60.9)	
NM, n (%)	5 (16.7)	625 (39.6)		2 (7.4)	604 (40.1)		8 (53.3)	658 (39.1)	
Unknown, n	4	202		5	187		0	0	

dMMR, deficient mismatch repair; M, mutated; MMR, mismatch repair; MSI, microsatellite instability; MSS; microsatellite stability; NM, nonmutated; pMMR, proficient mismatch repair; WHO, World Health Organization.

<sup>a</sup>Fisher exact test; Pearson  $\chi^2$  test.

**Supplementary Table 3.** Clinicobiological Characteristics Associated With *ERBB2* RNA Sequencing Expression Group in Pooled Population

Characteristic	ERBB2 RNA-seq expression group		P value <sup>a</sup>
	Intermediate (n = 2128)	Low (n = 717)	
Age			.12
<70 y, n (%)	1 717 (81.0)	561 (78.4)	
≥70 y, n (%)	402 (19.0)	155 (21.6)	
Unknown, n	9	1	
Gender			.2
Female, n (%)	933 (44.0)	296 (41.3)	
Male, n (%)	1 186 (56.0)	420 (58.7)	
Unknown, n	9	1	
WHO			.8
0, n (%)	1 628 (78.5)	547 (78.0)	
≥1, n (%)	447 (21.5)	154 (22.0)	
Unknown, n	53	16	
Tumor site			<.001
Proximal, n (%)	781 (37.4)	323 (45.9)	
Distal, n (%)	1 289 (61.8)	372 (52.9)	
Dual, n (%)	16 (0.8)	8 (1.1)	
Unknown, n	42	14	
Bowel obstruction and/or perforation			.065
Yes, n (%)	392 (18.5)	155 (21.6)	
No, n (%)	1 727 (81.5)	561 (78.4)	
Unknown, n	9	1	
pT			.007
pT1–3, n (%)	1 704 (80.5)	542 (75.7)	
pT4, n (%)	414 (19.5)	174 (24.3)	
Unknown, n	10	1	
pN			.051
pN1, n (%)	1 448 (68.3)	461 (64.4)	
pN2, n (%)	671 (31.7)	255 (35.6)	
Unknown, n	9	1	
Histopathology grading			<.001
G1–2, n (%)	1 815 (87.5)	562 (80.6)	
G3–4, n (%)	259 (12.5)	135 (19.4)	
Unknown, n	54	20	
RAS			.028
M, n (%)	910 (48.3)	337 (53.3)	
NM, n (%)	975 (51.7)	295 (46.7)	
Unknown, n	243	85	
BRAF			<.001
M, n (%)	194 (10.1)	103 (15.9)	
NM, n (%)	1 723 (89.9)	545 (84.1)	
Unknown, n	211	69	
MMR			.6
MSI/dMMR, n (%)	194 (9.5)	60 (8.8)	
MSS/pMMR, n (%)	1 847 (90.5)	620 (91.2)	
Unknown, n	87	37	
CMS			<.001
CMS1, n (%)	336 (15.8)	146 (20.5)	
CMS2, n (%)	958 (45.2)	190 (26.7)	
CMS3, n (%)	338 (15.9)	85 (11.9)	
CMS4, n (%)	488 (23.0)	291 (40.9)	
Unknown, n	8	5	

dMMR, deficient mismatch repair; M, mutated; MMR, mismatch repair; MSI, microsatellite instability; MSS, microsatellite stability; NM, nonmutated; pMMR, proficient mismatch repair; WHO, World Health Organization.

<sup>a</sup>Pearson  $\chi^2$  test.