Breast Cancer Research and Treatment (2019) 174:55–63
https://doi.org/10.1007/s10549-018-5049-7

PRECLINICAL STUDY

Identification, clinical-pathological characteristics and treatment outcomes of patients with metastatic breast cancer and somatic human epidermal growth factor receptor 2 (*ERBB2*) mutations

Lynn Jongen[1] · Giuseppe Floris[2,3] · Bram Boeckx[4,5] · Dominiek Smeets[4,5] · Diether Lambrechts[4,5] · Sara Vander Borght[3,4] · Annouschka Laenen[6] · Grace Mann[7] · Richard E. Cutler Jr.[7] · Alshad S. Lalani[7] · Patrick Neven[1,8] · Hans Wildiers[1,9]

Received: 3 November 2018 / Accepted: 13 November 2018 / Published online: 19 November 2018

Abstract

Purpose The human epidermal growth factor receptor 2 (*ERBB2*) may harbour somatic mutations that drive breast tumorigenesis. Here, we study prevalence, tumour characteristics and disease outcome of *ERBB2* mutations in a large unselected cohort of metastatic breast cancer (mBC) patients.

Methods We retrospectively included all mBC patients with sufficient primary breast tumour, diagnosed between 2000 and 2015 ($n=775$). Genomic DNA was subjected to a targeted-resequencing assay to identify hotspot mutations in exon 8, 17, 19, 20, and 21 of *ERBB2*. We studied demographics, tumour characteristics, median distant disease-free survival (DDFS), using a time-to-event analysis and time to progression (TTP) and overall survival (OS) upon metastasis, using Kaplan–Meier and log-rank statistics to assess differences between *ERBB2*-mutation statuses.

Results *ERBB2* mutations were observed in 1.8% of the samples (13/721). Patient and tumour characteristics were independent of *ERBB2* mutations. Luminal *ERBB2*-mutated ($ERBB2^{mut+}$) cases ($n=5$) had a shorter DDFS than $ERBB2^{mut-}$ cases (median DDFS 0.8 vs. > 4.0 years, $p=0.02$). ER-positive $ERBB2^{mut+}$ patients who received an aromatase inhibitor (AI) as first-line treatment (stage IV disease) had a worse TTP vs. $ERBB2^{mut-}$ patients ($n=3$ vs. 156; median TTP 103 vs. 311 days, $p=0.04$). OS for all subtypes was lower for $ERBB2^{mut+}$ vs. $ERBB2^{mut-}$ cases ($n=11$ vs. 669; median OS 1.1 vs. 2.3 years, $p=0.46$).

Conclusion $ERBB2^{mut+}$ are rare in patients in whom mBC developed and no evidence was found for an association with specific types of BC or patient characteristics, although outcomes of $ERBB2^{mut+}$ carriers might be worse. The latter, however, needs to be validated in larger populations.

Keywords Breast cancer · Somatic mutations · Metastasis · Human epidermal growth factor receptor 2

Abbreviations

AI	Aromatase inhibitor
DDFS	Distant disease-free survival
$ERBB2^{amp+/-}$	Human epidermal growth factor receptor 2 amplification positive/negative
$ERBB2^{mut+/-}$	Human epidermal growth factor receptor 2 mutation positive/negative
FFPE	Formalin fixed paraffin embedded
IDC	Invasive ductal carcinoma
ILC	Invasive lobular carcinomas
mBC	Metastatic breast cancer
OS	Overall survival
PR	Progesterone receptor
RCB	Residual cancer burden
SNVs	Single nucleotide variants
TILs	Tumour infiltrating lymphocytes
TNBC	Triple-negative BC
TTP	Time to progression
UHL	University Hospitals Leuven

Lynn Jongen and Giuseppe Floris have contributed equally to this work.

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s10549-018-5049-7) contains supplementary material, which is available to authorised users.

✉ Hans Wildiers
hans.wildiers@uzleuven.be

Extended author information available on the last page of the article

ER	Oestrogen receptor
ERBB2	Human epidermal growth factor receptor 2

Introduction

Patients with metastatic breast cancer (mBC) are generally incurable and novel treatment options are urgently needed. Targeted therapies against the human epidermal growth factor receptor 2 (ERBB2, also known as HER2) have improved patients outcomes significantly in the last decade in tumours with ERBB2 protein overexpression (*ERBB2*$^{amp+}$) due to gene amplification [1, 2]. Besides being amplified at the genetic level, the *ERBB2* receptor can also be mutated, which possibly may lead to altered receptor function and tumour cell activation [3]. Neratinib, an irreversible pan-ERBB tyrosine kinase inhibitor, has been demonstrated not only to potently inhibit breast cancers that are *ERBB2*$^{amp+}$, but also those with activating *ERBB2* mutations (with or without amplification) [3]. Therefore, activating *ERBB2* mutations are a potential new therapeutic target. The recent SUMMIT trial investigated the efficacy of neratinib in various *ERBB2*$^{mut+}$ cancers and found the greatest antitumor effect in breast cancer (median progression free survival of 3.5 months with neratinib monotherapy in patients with mBC carrying a somatic *ERBB2* mutation) [4].

ERBB2 mutations are rare with a frequency of 2% in early breast cancer patients [5]. It has been suggested that *ERBB2* mutations may be more common in patients who develop mBC but data are scarce. According to current literature, the prevalence of *ERBB2* mutations in breast cancer patients is about 2.6% (338/12,905, pooled data). The large majority of these cases were early stage breast cancers, but biopsies from mBC were also included [5]. The most frequent *ERBB2* mutations observed in literature in breast cancer are (in descending order of appearance) L755S (24% of *ERBB2*-mutated tumours), Val777Leu (13.7%), Asp769His and Asp769Tyr (both 7.8%), etc. Notably, these *ERBB2* hotspot mutations were all located in the *ERBB2* kinase domain [5]. In vitro and in vivo experiments have proven that these activating mutations lead to a constitutively-active tyrosine kinase receptor compared to *ERBB2*$^{mut-}$ [3]. However, not all *ERBB2* mutations are biologically characterised.

Although ERBB2 tyrosine kinase inhibitors have been developed for the treatment of mBC patients carrying an *ERBB2* mutated tumour, little is known about the prevalence and the clinical implications of *ERBB2* mutations in this setting. We, therefore, established a large retrospective cohort study with the following objectives: (1) to evaluate the frequency of *ERBB2* mutations in a large cohort of patients in whom mBC developed, (2) to understand the demographic, clinical, pathological and patient characteristics associated with *ERBB2* mutations and (3) to characterize treatment responses and outcomes to standard therapies in patients with *ERBB2*$^{mut+}$ versus *ERBB2*$^{mut-}$ tumours.

Methods

Patient selection

This retrospective study included patients with mBC diagnosed at the Multidisciplinary Breast Centre of University Hospitals Leuven (UHL). The main inclusion criterion was diagnosis of mBC between January 1st, 2000 and July 31, 2015. Both primary (metastases at first breast cancer diagnosis) and secondary mBC (developing metastatic disease after early breast cancer) were included. All breast cancer types were eligible. Further inclusion criteria were availability of primary invasive tumour tissue, and availability of clinical follow-up data, which stopped at June 2017. The study was conducted in accordance with the provision of the Declaration of Helsinki and Good Clinical Practice guidelines and approved by the Ethics Committee of the UHL in Belgium before the study started. Patients were only included if they provided a written informed consent at time of diagnosis for future research on their resected tumour tissue. In Fig. 1, a flowchart is displayed of the patient selection.

ERBB2 mutation screening

Genomic DNA was extracted from formalin fixed paraffin embedded (FFPE) primary tumour tissue containing > 10% tumour tissue. Only DNA samples with a total yield > 20 ng DNA were considered. DNA was subjected to targeted resequencing using an amplicon-based assay covering 5 exons (exons 8, 17, 19, 20, and 21) known to accumulate *ERBB2* hotspot mutations (Supplementary Table 1). This assay was able to detect four types of predefined or annotated aberrations of interest, including single nucleotide variants (SNVs), small insertions, deletions and indels (complex deletion/insertions) within *ERBB2* (Supplementary Table 1).

The *ERBB2* hotspot mutation resequencing assay was validated (in a blinded fashion) on 16 commercial cell lines (Horizon Discovery, Cambridge, UK) each harbouring a different *ERBB2* mutation. We further optimized our design by using positive control sample sets of five serial diluted (ranging from 1/5 to 1/250) mutated and wild-type cell lines (each of these harboured a different *ERBB2* mutation in one of the 5 exons), and on 48 FFPE tumour samples some of which yielded tumour DNA of inferior quality. In the dilution series with decreasing amounts of input DNA, we were able to detect the different *ERBB2* mutations down to about 10fM of input DNA in both the commercial cell lines and the plasmids.

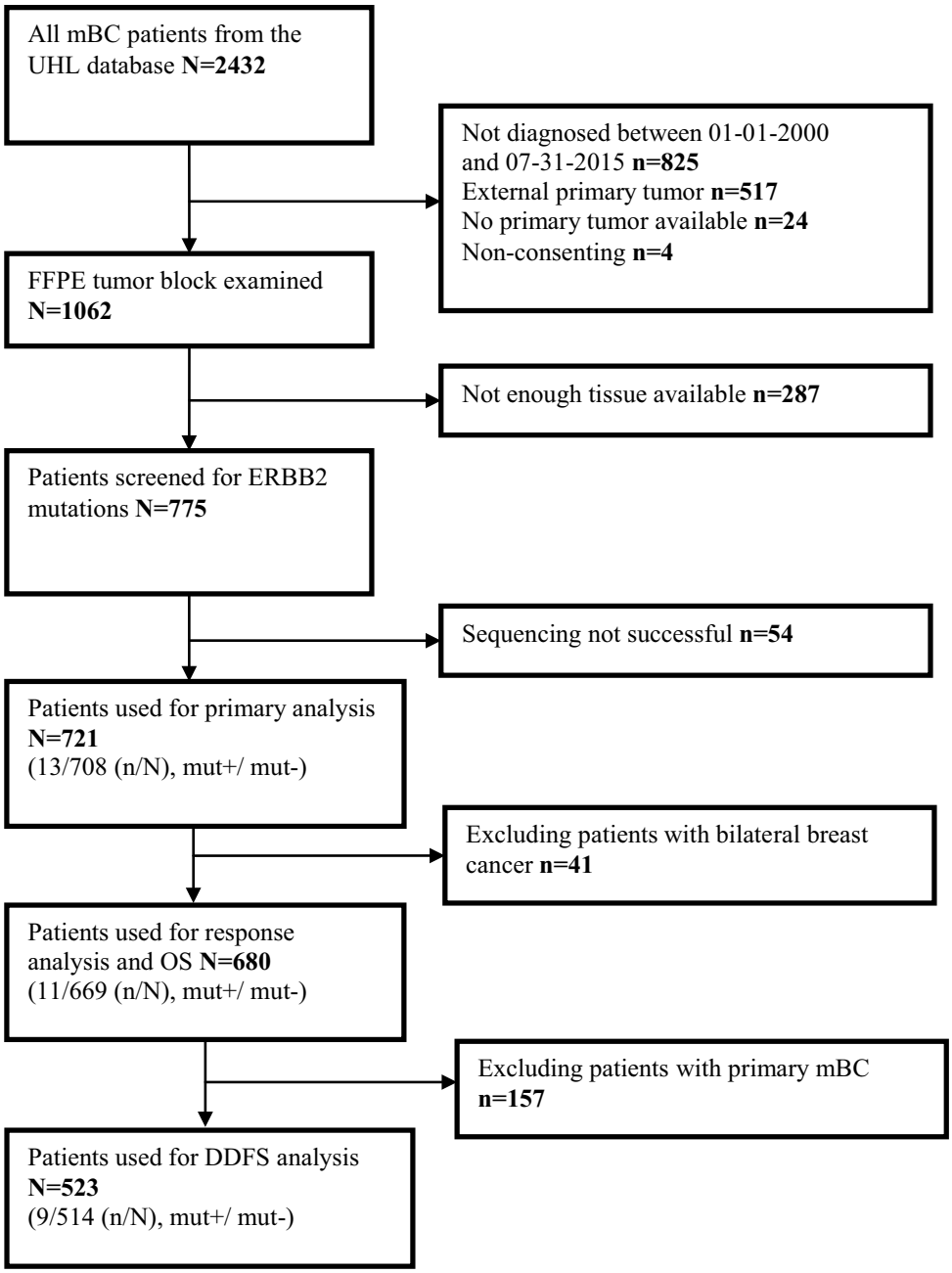

Fig. 1 Flowchart of patient selection. In total, we investigated 721 patients with/without an *ERBB2* mutation. For the analysis of the time to progression on treatment in the metastatic setting and the overall survival (OS), we excluded patients with bilateral breast cancer ($n=680$). In addition, we excluded the patients with primary metastatic breast cancer (mBC) for the distant disease free survival (DDFD) analysis ($n=523$). mBC: metastatic breast cancer, UHL: University Hospitals Leuven, FFPE: formalin fixed paraffin embedded, ERBB2: human epidermal growth factor receptor 2

All samples were re-sequenced at an average coverage of $>500\times$ per exon with a median spread factor of 8.83, which was defined as the difference in coverage between the lowest and highest covered exon. All samples were screened for *ERBB2* mutations at least two times with two independent experiments. The assay detected all *ERBB2* mutations with high sensitivity, i.e., 2% or 3x the standard deviation of the background noise (Illumina HiSeq 2500). We only considered mutations positive if they were confirmed in at least 2 independent experiments and were at least once observed with an allelic frequency of $\geq 5\%$.

Central pathological revision

A dedicated breast pathologist (GF) performed central pathological revision of the $ERBB2^{mut+}$ cases. For all these cases, histology was re-examined and new staining's were performed to confirm the expression level of ER, PR, $ERBB2^{amp+}$ (amplification with IHC and FISH), KI-67 (proliferation marker). Surrogate intrinsic molecular subtypes were defined as luminal (oestrogen receptor (ER)-positive with $>1\%$ positive cells, and $ERBB2^{amp-}$ according to IHC and/or FISH), triple-negative breast cancer (ER-negative,

progesterone receptor (PR)-negative, *ERBB2*$^{amp-}$) and *ERBB2*$^{amp+}$ (*ERBB2*$^{amp+}$according to FISH). On H&E, tumour infiltrating lymphocytes (TILs) were scored according to the International TILs Working Group [6, 7]. In post-neoadjuvant resection specimens, the residual cancer burden (RCB) score was calculated as recommended by Symmans et al. [8].

Response to treatment and outcomes

To evaluate the response to standard therapy once metastatic, we used the time to progression (TTP) on each metastatic treatment line in the metastatic setting. To make the data more interpretable, treatments were categorized into one of the following groups: tamoxifen, aromatase inhibitor (AI), fulvestrant, ERBB2-targeted therapy in combination with chemotherapy, chemotherapy, other (e.g. combination of the groups, everolimus and exemestane or chemotherapy followed by endocrine therapy). TTP was determined in days from start of treatment till end/start of new treatment, due to progressive disease.

The outcome after standard of care therapy was investigated only for patients with unilateral breast cancer at diagnosis. Patients with synchronous bilateral breast cancer were excluded from the analysis, because of the uncertainty about the origin of the metastatic site due to the lack of collected tissue from the metastases. For the patients who developed secondary metastases, the distant disease-free survival (DDFS, the time between primary diagnosis and metastasis) in years was investigated. Next, we evaluated overall survival (OS, the time between metastasis and death) in years.

Statistical analysis

Comparisons between two groups were performed using Chi square test for categorical variables or Mann–Whitney U test for continuous variables. For the survival analysis, patients with bilateral breast cancer were excluded. OS was estimated using the Kaplan–Meier method, and the log-rank test was used for assessing differences between groups. If patients had no event at the database lock (June 2017) for the final analysis, they were censored at the last follow-up visit at the hospital. All tests were two-sided, a 5% significance level was assumed for all tests. Analyses have been performed using SAS software (version 9.4 of the SAS System for Windows).

Results

Identification of *ERBB2* mutations in primary tumour of patients with mBC

Of all 2432 mBC patients seen in Leuven, 775 fulfilled our pre-defined inclusion criteria and had sufficient tissue available for targeted re-sequencing with our *ERBB2* hotspot mutation panel (Fig. 1). Of these, we could successfully sequence 721 tumour tissues and we identified 14 *ERBB2* somatic mutations in 13 patients with mBC. One of these 13 patients carried two *ERBB2* mutations. Overall, this results in an *ERBB2* mutation prevalence of 1.8% (13/721).

The clinical information of the 13 patients with an *ERBB2*$^{mut+}$ tumour is described in Table 1. Interestingly, in patients with synchronous bilateral breast cancer ($n=41$), after screening for *ERBB2* mutations in both sides, we found two patients carrying *ERBB2*$^{mut+}$ tumours only at one side. Unfortunately, for these patients, no metastatic tissue was available. It was, therefore, not possible to determine if the metastases were derived from the *ERBB2*$^{mut+}$ or *ERBB2*$^{mut-}$ tumour.

Several of the *ERBB2* mutations were observed in more than one tumour. Particularly, *ERBB2* mutations Leu755Ser ($n=5$) and Arg678Gln ($n=5$) and Ser310Phe ($n=2$) were recurrently detected. The majority of *ERBB2* mutations were observed in the *ERBB2* kinase domain (50%), in the juxtamembrane (36%) or in the extracellular domain (14%).

Pathological and patient characteristics of *ERBB2*$^{mut+}$ patients

Patients with an *ERBB2*$^{mut+}$ tumour exhibited similar demographics (age at diagnosis, bilaterality, BMI and menopausal status) and tumour characteristics (histological subtype, grade, pTN, ER/PR/*ERBB2*^{amp} status, number of foci as well as lymph-vascular invasion) as breast tumours not carrying an *ERBB2* mutation (Supplementary Tables 2 and 3). Additionally, *ERBB2* mutation status (yes vs. no) did not differ for the first site of metastasis (data not shown). However, we did observe a non-significant enrichment in the ILC type in the *ERBB2*$^{mut+}$ tumours as compared to the *ERBB2*$^{mut-}$ ones ($4/13=31\%$ vs. $97/695=14\%$, respectively; $p=0.09$). Besides 6 IDC, we found one apocrine carcinoma, and in two other tumours, a micro-papillary component and mucinous component was observed together with the IDC component (< 50% and 50% of the whole tumour, respectively).

Two patients had multifocal tumours, in patient 11 (Table 1) the two foci showed the same morphology and receptor status, therefore, only one focus was screened for *ERBB2* mutations. Patient 10 (Table 1) was initially reported to have a unifocal IDC, but at the pathological revision, a second micro-invasive (i.e. < 1 mm) focus of ILC was found in a zone adjacent to lobular carcinoma in situ, classic type. In this case, only the invasive ductal carcinoma (IDC) was screened for *ERBB2*-mutations. No grade 1 tumours were present in our cohort of *ERBB2*$^{mut+}$ breast carcinomas, the majority had tumours > 2 cm, lymph-vascular invasion and lymph nodes metastasis were observed in about 50% of the cases.

Table 1 Overview of clinical-pathological characteristics and response on treatment in the metastatic setting in patients with an *ERBB2*$^{mut+}$ tumor

Patient	BC status at diagnosis	Type	Grade	pTN	Molecular Subtype	ER	PR	ERBB2^{amp} IHC	*ERBB2*^{amp}FISH	Ki-67 (%)
1	EBC	IDC	3	pT2N2a	ERBB2	+	−	3+	+	10
2	EBC[a]	IDC	3	pT2N0(i-)	ERBB2	+	−	3+	+	35
3	pmBC	IDC	3	NA	TNBC	−	−	0	NA	40
4	EBC	IDC[d]	3	pT1cN0(i-)	TNBC	−	−	0	NA	15
5	NBC[b]	Pleiomorf ILC	2	ypT3N1	Lum	+	−	2+	NA	5
6	EBC[a]	IDC	2	pT1cN0(i-)	Lum	+	+	1+	−	3
7	NBC[c]	ILC	2	NA	Lum	+	+	2+	−	20
8	EBC	ILC	3	pT3N2a	ERBB2	+	+	2+	+	10
9	EBC	Mixed IDC + mucinous	2	pT2N1a	Lum	+	+	1+	−	10
10	EBC	IDC	3	pT2N0	Lum	+	+	0	NA	5
11	EBC	ILC	2	pT2N3a	Lum	+	+	1+	NA	2
12	EBC	Apocrine carcinoma	3	pT2N0	ERBB2	−	−	3+	+	25
13	pmBC	IDC	3	NA	Lum	+	+	1+	−	10

Patient	TILs (%)	LVI	PNI	No Foci	Exon	*ERBB2* mutation	Allelic freq. (%)	TTP in days on 1st line	TTP in days on 2nd line
1	50–60	Yes	No	1	8	Ser310Phe	19	AI:31	CT:165
2	30–40	No	No	1	8	Ser310Phe	22	/	
3	30	No	NA	1	17	Arg678Gln	6	CT:203	CT:282[g]
4	10–20	Yes	No	1	17	Arg678Gln	7	NT	
5	< 1	No	No	1	17	Arg678Gln	9	CT:158	Tam:29
6	10	Yes	No	1	17	Arg678Gln	9	/[g]	
7	1	No	No	1	17	Arg678Gln	59	AI:103	
					19	Leu755Ser	58		
8	0	Yes	No	1	19	Leu755Ser	77	Other:13	
9	5	No	No	1	19	Leu755Ser	24	Tam:1047	AI:221
10	5	Yes	No	2[f]	19	Leu755Ser	8	AI:115	CT:156
11	5	No	No	2[e]	19	Leu755Ser	23	Tam:847	AI:80
12	30	Yes	No	1	20	Val777Leu	43	ERBB2:425 [g]	
13	< 1	No	NA	1	20	Pro780_Tyr781 Ins GSP	32%	Tam:34	

EBC early breast cancer, *NBC* neoadjuvant breast cancer, *pmBC* primary metastatic breast cancer, *ER* oestrogen receptor, *PR* progesterone receptor, *ERBB2*^{amp} human epidermal growth factor receptor 2 amplification, *TILs* tumour infiltrating lymphocytes, *Ki-67* proliferation marker, *LVI* Lymph-vascular invasion, *PNI* perineural invasion, *TTP* time to progression in days, *IDC* invasive ductal carcinoma, *ILC* invasive lobular carcinoma, *AI* aromatase inhibitor, *CT* chemotherapy, *Tam* tamoxifen, *ERBB2* ERBB2 targeted therapy, *NT* no therapy in the metastatic setting since the patient deceased before therapy could start, *NA* not applicable since these patients had bilateral breast cancer and it remained unclear which of both tumours caused the metastases

[a]Bilateral breast cancer: only one of both tumours had an *ERBB2* mutation. These tumours were included in the analyses on tumour characteristics, but not in the outcome results. Unfortunately, of these bilateral cases, no metastatic tissue was available to determine presence of *ERBB2* mutations

[b]Residual cancer burden: RCB-II after neoadjuvant chemotherapy

[c]Initially unclear whether or not metastases at diagnosis, so upfront tamoxifen was started. The patient developed overt metastases during tamoxifen

[d]This tumour had a minor micropapillary component

[e]Both foci had the same pathology

[f]Only at the revision of the pathology a small focal ILC was discovered, only the IDC was screened for *ERBB2* mutations

[g]The patient is still alive

Notably, we observed *ERBB2* mutations in all breast cancer subtypes: luminal ($n=7$), triple-negative ($n=2$), as well as in $ERBB2^{amp+}$ ($n=4$) breast cancer. We compared the patient demographic and clinic-pathological characteristics of patients with $ERBB2^{mut+}$ vs. $ERBB2^{mut-}$ (Supplementary Tables 4 and 5) per molecular surrogate subtype but we did not find any statistically significant difference, although the numbers were small.

Subsequently, we measured on H&E the amount of tumour infiltrating lymphocytes (TILs) in the stroma surrounding the tumour nests. Luminal tumours showed very low level of TILs, while we observed higher TILs only in $ERBB2^{amp+}$ or TNBC subtypes in a fashion similar to that described in $ERBB2^{mut-}$ breast tumours. We concluded that *ERBB2* mutations in breast cancers are unlikely to be highly immunogenic (Table 1). Two patients (patient 5 and 7; Table 1) with an $ERBB2^{mut+}$ primary tumour received upfront systemic therapy. Patient 5 received neo-adjuvant doxorubicin and docetaxel, and after therapy, we observed partial pathological response with a residual cancer burden (RCB) class II (i.e. still 10% measurable overall cancer cellularity in the tumour bed area, of which 90% was invasive carcinoma) [8, 9]. Patient 7 received upfront tamoxifen (initial uncertainty about presence of metastases at diagnosis), but was diagnosed with metastatic disease during upfront tamoxifen. Therefore, this patient did not undergo any surgery.

Distant disease-free survival of $ERBB2^{mut+}$ tumours in patients with mBC

The median follow-up for all the patients with unilateral early breast cancer was 5.8 years ($n=523$; range = 0.4–16.4 years), while for the luminal tumours, the median follow-up was 7.2 years ($n=333$; range = 0.6–16.4 years). Median DDFS for all surrogate intrinsic molecular subtypes was 1.4 years (IQR 0.8; 3.2) for patients with an $ERBB2^{mut+}$ tumour ($n=9$) compared to 2.9 years (IQR 1.6; 6.1) in patients with an $ERBB2^{mut-}$ tumours ($n=514$) ($p=0.10$). In the patients with luminal tumours, we observed a significantly shorter DDFS in $ERBB2^{mut+}$ ($n=5$) vs. $ERBB2^{mut-}$ ($n=328$) cases (median DDFS 0.8 (IQR 0.8; 1.6) vs. 4.0 years (IQR 2.3; 7.1), $p=0.02$). For other subtypes, there was no significant difference in DDFS in $ERBB2^{mut+}$ vs. $ERBB2^{mut-}$ cases.

Treatment outcome during standard therapy in $ERBB2^{mut+}$ patients

ER-positive patients with unilateral $ERBB2^{mut+}$ tumours who received an AI as first line therapy ($n=3$) had a worse TTP compared to $ERBB2^{mut-}$ patients ($n=3$ vs. $n=156$; median TTP 103 vs. 311 days, $p=0.04$). Similar findings were observed when considering mBC patients in which AI was delivered during any treatment line ($n=8$ vs. $n=376$, median TTP 74 vs. 213 days, $p=0.01$). TTP was not significantly different for other standard therapies, but numbers were small.

Overall survival of patients with an *ERBB2* mutation

When assessing differences in OS for the 680 patients with survival data available, we observed that OS was not significantly different in $ERBB2^{mut+}$ ($n=11$) vs. $ERBB2^{mut-}$ ($n=669$) cases (median OS 1.1 vs. 2.3 years; $p=0.46$) (Table 2). Among patients with luminal tumours, OS was shorter for patients with $ERBB2^{mut+}$ carrying (median OS 1.0 vs. 2.9 years; $n=6$ vs. $n=431$, respectively, for $ERBB2^{mut+}$ vs. $ERBB2^{mut-}$ carriers), although this did not reach statistical significance ($p=0.07$). In addition, the other subtypes of BC did not demonstrate any significant difference in OS between the $ERBB2^{mut+}$ patients vs. $ERBB2^{mut-}$ patients, although numbers were relatively small (Table 2).

Discussion

To our knowledge, this is the first study that investigates the natural history of a large cohort of patients who developed mBC by the presence of an *ERBB2* mutation in the primary tumour. We demonstrated that *ERBB2* mutations are rare in mBC with a frequency of 1.8%. Previous studies observed similar frequencies of *ERBB2* mutations in patients with early breast cancer [5, 10]. Our study suggests that *ERBB2* mutations are not more frequent in patients who developed

Table 2 Median overall survival (OS) in years in the subgroup of unilateral cases ($n=680$) between diagnosis of metastatic breast cancer and death of any cause for all subtypes combined and per surrogate intrinsic molecular subtype according to *ERBB2* mutation

Subtype	$ERBB2^{mut-}$	$ERBB2^{mut+}$	*p*-Value
All subtypes			
Patients (*n*)	669	11	
Median OS (year)	2.3	1.1	0.46
Luminal-like			
Patients (*n*)	431	6	
Median OS (year)	2.9	1.0	0.07
ERBB2-like			
Patients (*n*)	112	3	
Median OS (year)	2.3	1.4	0.33
TNBC-like			
Patients (*n*)	117	2	–
Median OS (year)[a]	1.0	–	

n number of patients, *y* year

[a]Not enough patients for analysis

mBC. We only screened for *ERBB2* mutations in the primary tumour tissue of our metastatic cohort because of lack of metastatic tissue. One may argue that the mutations can occur at a later stage, but Ross et al. [11] showed in a recent study that *ERBB2* mutations are not more common in metastatic tissue compared to primary tissue.

We developed a research-use-only targeted *ERBB2* mutation NGS-based assay covering five exons of *ERBB2*. These five exons were selected as they are known to contain the most frequent hot-spot mutations in the *ERBB2*. With this NGS assay, we were able to detect four types of aberrations including, single nucleotide variants, small insertions, deletions and indels (complex deletions/insertions) within these exons of *ERBB2*. More specific, we observed five different *ERBB2* mutations (Arg678Gln, Leu755Ser, Ser310Phe, Val-777Leu, and Pro780_Tyr781insGSP) across the five exons we screened. Leu755Ser was the most frequent mutation in our cohort, which is consistent with literature [5].

Our data suggest that $ERBB2^{mut+}$ tumour have similar demographic, clinical and tumour characteristics when compared with wild-type patients. There may be a slight increase in lobular breast cancers (4/13 in our series) which is also consistent with literature [12], and another recent study found that 18% of the CDH1-mutated ILC have an *ERBB2* mutation [13].

We evaluated also the lymphocytic stromal infiltrate in the patients with an $ERBB2^{mut+}$ tumour using the TILs scoring method [6]. Overall, the amount of stromal TILs was not particularly influenced by the presence of *ERBB2* mutations. On the contrary, we observed that the TILs scores were rather influenced by the type of surrogate intrinsic molecular subtype in a fashion consistent to that already reported in the literature [14, 15]. Therefore, we speculate that most likely the presence of an *ERBB2* mutations by itself does not seem to influence consistently the immunological status of breast carcinomas.

When all molecular subtypes were compared, no difference was observed in survival with an $ERBB2^{mut+}$ tumour compared to $ERBB2^{mut-}$ tumour. However, in the luminal subtype, we observed a shorter DDFS and a shorter OS in the patients with an $ERBB2^{mut+}$*tumour*. In contrast, a recent study examining combined data of three clinical trials with ER-positive patients did not found *ERBB2* mutations to be prognostic [10] so this needs further confirmation. Interestingly, in our study, we observed that ER-positive patients had a worse TTP on an AI and a poorer survival. This ER-ERBB2 crosstalk was also seen by two abstracts suggesting that *ERBB2* mutations can confer endocrine resistance in Luminal BC, and that this resistance mechanism can be effectively reversed by dual blockade of ER and ERBB2 [16, 17]. This might suggest that *ERBB2* mutations play a role in the resistance mechanism of an AI in patients with an ER-positive primary tumour.

Preclinical research showed that most *ERBB2* mutations are activating mutations [3, 18, 19]. Efficacy of an irreversible pan-ERBB tyrosine kinase inhibitor is currently tested in patients with solid tumours (e.g. breast cancer and lung cancer) with amongst others activating *ERBB2* mutations. Hyman et al. [4] showed in the SUMMIT trial (NCT01953926) the importance of *ERBB2* mutations in cancer patients (including but not restricted to breast cancer). In that trial, they observed an objective response rate of 24% for patients with $ERBB2^{mut+}$ mBC with acceptable toxicity profile.

There are several limitations to this study. First, the small number of patients with an *ERBB2* mutation resulted that this study should be considered explorative and hypothesis-generating. Although, we attempted to sample a very large sample of mBC patients ($n = 1062$), there were a significant number ($n = 287$) of dropouts because of insufficiently available primary tumour tissue and 54 because of unsuccessful sequencing. Second, primary tumour tissue was investigated since the availability of sufficient and good quality metastatic tumour tissue was not available. Nevertheless, also the primary tumour tissue was very relevant for our purpose, i.e. identify the characteristics and natural history of patients with *ERBB2* mutations. Interestingly, Liu et al [20] showed that in invasive ductal carcinoma *ERBB2* mutations were significantly associated with metastasis. Additionally, Schrijver et al. [21] showed that 45% of individual variants (45% of the non-synonymous variants) were shared between primary tumour and metastasis, with a range of 23%-100% for non-synonymous variants per patient. Further research is necessary to investigate the comparison with primary tumour tissue and paired metastatic tissue and ctDNA samples. Third, the extracted DNA did not reach quality standards for the NGS analysis in 7% (54/775) of the patients which is not unusual in retrospective studies based on the use of archive material. This issue points out the importance of stringent quality standard during the pre-analytical phases in handling fresh tumour material.

We initially aimed to investigate two other objectives. First, patient with an $ERBB2^{mut+}$ tumour were screened for the co-occurrence of other oncogenes including the *ERBB2* with a next generation sequencing assay using a 97-gene panel. Unfortunately, the residual DNA concentration was too low and if there was enough DNA, the coverage was not sufficient from the hybridisation capture method to evaluate the co-occurrence with *ERBB2* (data not shown). Second, we aimed to check if *ERBB2* mutations can be detected from plasma samples collected at baseline and metastatic tumour tissue. ctDNA could not be isolated from the baseline plasma samples since they were not collected in the ideal setting for ctDNA. From the four matching metastatic tumour tissues that were tested, DNA was only enough for three samples,

however, the sequencing failed; probably because the samples were too fragmented.

In conclusion, *ERBB2* somatic mutations are rare in patients who developed mBC, and occur in all intrinsic molecular subtypes. Besides the impression that ILC histological subtype are slightly more frequent among the $ERBB2^{mut+}$ breast tumours, we observed no difference in demographic, clinical, or pathological characteristics compared to $ERBB2^{mut-}$ cases. The outcome in terms of prognosis and response to aromatase inhibition in luminal tumours suggest a possible mechanism of resistance; however, this observation needs validation in larger series.

Acknowledgements The authors would like to thank Sarah Cumps and Wilfried Versin for their training and technical assistants in histology at the Department of Imaging and Pathology, Translational Cell & Tissue Research of the KU Leuven. We further thank Gilian Peuteman, Evy Vanderheyden, and Thomas Van Brussel from the VIB Center for Cancer Biology, laboratory of Translational Genetics, for performing the DNA extractions, and the sequencing. We would like to acknowledge Chantal Remmerie, from the Multidisciplinary Breast Centre of University Hospitals Leuven for setting up the database of the patients with breast cancer. The computational resources used in this work were provided by the Flemish Supercomputer Center (VSC), funded by the Hercules Foundation and the Flemish Government, Department of Economy, Science and Innovation (EWI).

Funding The work was supported by Puma Biotechnologies Inc..

Compliance with ethical standards

Conflict of interest HW is recipient of The Research Foundation - Flanders (FWO). HW received consulting fees, travel support, and research support from Puma Biotechnologies, Inc (all provided to his institute), and he received speaker's fees, consulting fees, travel support, and research support from Roche (all provided to his institute). LJ received a grant from Puma Biotechnologies, Inc. GM, AL, and RC received salary and have ownership interest in Puma Biotechnologies, Inc. PN received speaker's fees, consulting fees, travel support, and research support from Roche (all provided to his institute). PN is on the advisory board of Novartis, AstraZeneca, Lilly, and Pfizer (all consulting fees are provided to his institute).

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional committee (University Hospitals Leuven, Belgium) and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Patients were only included if they provided a written informed consent at time of diagnosis for future research on their resected tumour tissue. This article does not contain any studies with animals performed by any of the authors.

References

1. Perez EA, Romond EH, Suman VJ et al (2014) Trastuzumab plus adjuvant chemotherapy for human epidermal growth factor receptor 2-positive breast cancer: planned joint analysis of overall survival from NSABP B-31 and NCCTG N9831. J Clin Oncol 32:3744–3752
2. Gianni L, Dafni U, Gelber RD et al (2011) Treatment with trastuzumab for 1 year after adjuvant chemotherapy in patients with HER2-positive early breast cancer: a 4-year follow-up of a randomised controlled trial. Lancet Oncol 12:236–244
3. Bose R, Kavuri SM, Searleman AC et al (2013) Activating HER2 mutations in HER2 gene amplification negative breast cancer. Cancer Discov 3:224–237
4. Hyman DM, Piha-Paul SA, Won H et al (2018) HER kinase inhibition in patients with HER2- and HER3-mutant cancers. Nature 554:189–194
5. Petrelli F, Tomasello G, Barni S et al (2017) Clinical and pathological characterization of HER2 mutations in human breast cancer: a systematic review of the literature. Breast Cancer Res Treat 166:339–349
6. Salgado R, Denkert C, Demaria S et al (2015) The evaluation of tumor-infiltrating lymphocytes (TILs) in breast cancer: recommendations by an International TILs Working Group 2014. Ann Oncol 26:259–271
7. Hendry S, Salgado R, Gevaert T et al. Assessing tumor-infiltrating lymphocytes in solid tumors: a practical review for pathologists and proposal for a standardized method from the international immunooncology biomarkers working group: part 1: assessing the host immune response, TILs in invasive breast carcinoma and ductal carcinoma in situ, metastatic tumor deposits and areas for further research. Adv Anat Pathol 2017; 24: 235–251
8. Symmans WF, Peintinger F, Hatzis C et al (2007) Measurement of residual breast cancer burden to predict survival after neoadjuvant chemotherapy. J Clin Oncol 25:4414–4422
9. Symmans WF, Wei C, Gould R et al (2017) Long-term prognostic risk after neoadjuvant chemotherapy associated with residual cancer burden and breast cancer subtype. J Clin Oncol 35:1049–1060
10. Griffith OL, Spies NC, Anurag M et al (2018) The prognostic effects of somatic mutations in ER-positive breast cancer. Nat Commun 9:3476
11. Ross JS, Gay LM, Wang K et al (2016) Nonamplification ERBB2 genomic alterations in 5605 cases of recurrent and metastatic breast cancer: An emerging opportunity for anti-HER2 targeted therapies. Cancer 122:2654–2662
12. Deniziaut G, Tille JC, Bidard FC et al (2016) ERBB2 mutations associated with solid variant of high-grade invasive lobular breast carcinomas. Oncotarget 7:73337–73346
13. Ross JS, Wang K, Sheehan CE et al (2013) Relapsed classic E-cadherin (CDH1)-mutated invasive lobular breast cancer shows a high frequency of HER2 (ERBB2) gene mutations. Clin Cancer Res 19:2668–2676
14. Loi S, Michiels S, Salgado R et al (2014) Tumor infiltrating lymphocytes are prognostic in triple negative breast cancer and predictive for trastuzumab benefit in early breast cancer: results from the FinHER trial. Ann Oncol 25:1544–1550
15. Stanton SE, Adams S, Disis ML (2016) Variation in the incidence and magnitude of tumor-infiltrating lymphocytes in breast cancer subtypes: a systematic review. JAMA Oncol 2:1354–1360
16. Scaltriti M, Carmona FJ, Toska E et al (2016) Neratinib induces estrogen receptor function and sensitizes HER2-mutant breast cancer to anti-endocrine therapy. In 28th EORTC-NCI-AACR symposium on molecular targets and cancer therapeutics. Munich
17. Nayar U, Cohen O, Kapstad C et al. Acquired HER2 mutations in ER + metastatic breast cancer confer resistance to ER-directed therapies. In AACR Annual Meeting. Chicago: 2018
18. Wang T, Xu Y, Sheng S et al (2017) HER2 somatic mutations are associated with poor survival in HER2-negative breast cancers. Cancer Sci 108:671–677

19. Greulich H, Kaplan B, Mertins P et al (2012) Functional analysis of receptor tyrosine kinase mutations in lung cancer identifies oncogenic extracellular domain mutations of ERBB2. Proc Natl Acad Sci USA 109:14476–14481
20. Liu G, Zhan X, Dong C, Liu L (2017) Genomics alterations of metastatic and primary tissues across 15 cancer types. Sci Rep 7:13262
21. Schrijver W, Selenica P, Lee JY et al (2018) Mutation profiling of key cancer genes in primary breast cancers and their distant metastases. In: Cancer Res. United States: 2018 American Association for Cancer Research. 3112–3121

Affiliations

Lynn Jongen[1] · Giuseppe Floris[2,3] · Bram Boeckx[4,5] · Dominiek Smeets[4,5] · Diether Lambrechts[4,5] · Sara Vander Borght[3,4] · Annouschka Laenen[6] · Grace Mann[7] · Richard E. Cutler Jr.[7] · Alshad S. Lalani[7] · Patrick Neven[1,8] · Hans Wildiers[1,9]

1 Department of Oncology, KU Leuven - University of Leuven, Herestraat 49, 3000 Leuven, Belgium

2 Laboratory of Translational Cell & Tissue Research, Department of Imaging and Pathology, KU Leuven - University of Leuven, Herestraat 49, 3000 Leuven, Belgium

3 Department of Pathology, University Hospitals Leuven, Herestraat 49, 3000 Leuven, Belgium

4 Department of Human Genetics, KU Leuven - University of Leuven, Herestraat 49, 3000 Leuven, Belgium

5 Laboratory of Translational Genetics, VIB Center for Cancer Biology, Herestraat 49, 3000 Leuven, Belgium

6 Interuniversity Centre for Biostatistics and Statistical Bioinformatics, Leuven, Belgium

7 Puma Biotechnology, Inc., 10880 Wilshire Blvd., Suite 2150, Los Angeles, CA 90024, USA

8 Department of Gynaecology and Obstetrics, University Hospitals Leuven, KU Leuven - University of Leuven, Herestraat 49, 3000 Leuven, Belgium

9 Department of General Medical Oncology, University Hospitals Leuven, KU Leuven - University of Leuven, Herestraat 49, 3000 Leuven, Belgium