Integrated genomic and transcriptomic analysis of human brain metastases identifies alterations of potential clinical significance

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Abstract

Treatment options for patients with brain metastases (BM) have limited efficacy and the mortality rate is virtually 100%. Targeted therapy is critically under-utilised, and our understanding of mechanisms underpinning metastatic outgrowth in the brain is limited. To address these deficiencies, we investigated the genomic and transcriptomic landscapes of 36 BM (from breast, lung, melanoma and oesophageal cancers) using DNA copy-number analysis and exome- and RNA-sequencing. The key findings were: (1) identification of novel candidates with possible roles in brain metastasis development, including the significantly mutated genes DSC2, ST7, PIK3R1 and SMC5; and the DNA repair, ERBB/HER signalling, axon guidance and protein kinase-A signalling pathways. (2) Mutational signature analysis was applied to successfully identify the primary cancer type for two BM with unknown origins. (3) Actionable genomic alterations were identified in 31/36 BM (86%). In one case we retrospectively identified ERBB2-amplification representing apparent HER2 status conversion, then confirmed progressive enrichment for HER2-positivity across four consecutive metastatic deposits by IHC and SISH, resulting in the deployment of HER2-targeted therapy for the patient. (4) In the ERBB/HER pathway, ERBB2 expression correlated with ERBB3 ($r^2=0.496$; $p<0.0001$), and HER3 and HER4 were frequently activated in an independent cohort of 167 archival BM from seven primary cancer types: 57.6% and 52.6% of cases were phospho-HER3$^{Y1222}$ or phospho-HER4$^{Y1162}$ membrane-positive, respectively. The HER3 ligands NRG1/2 were barely detectable by RNAseq (with NRG1 (8p12) genomic loss in 63.6% breast cancer-BM), suggesting a microenvironmental source of ligand. In summary, this is the first study to characterise the genomic landscapes of BM. The data revealed novel candidates, potential clinical applications for genomic profiling of resectable BM, and highlight the possibility of therapeutically targeting HER3, which is broadly over-expressed and activated in BM independent of primary site and systemic therapy.

Keywords: brain metastasis, exome sequencing, RNA sequencing, genomic signature, targeted therapy, HER2, HER3
Introduction

The management of brain metastases (BM) is a critical area of unmet need [1] affecting over 100,000 cancer patients annually in the United States at a cost of more than $3B USD, largely for palliative care [2,3]. The incidence varies between primary cancer types (10-40% overall), being highest for lung, breast, renal cancers and melanoma [4,5]. Breast cancer patients with triple-negative, basal-like, claudin-low and HER2-positive (mostly ERBB2-amplified) tumours are at highest risk of brain relapse [6-9]. Current management strategies (surgery and whole-brain or stereotactic radiotherapy) are associated with neurological side effects, are not curative and limited to patients with one or a few small lesions [10-12]. Even with aggressive treatment, survival is often less than 12 months [13,14]. The incidence of BM is increasing due to more effective treatments for systemic (extracranial) disease, sophisticated surveillance imaging modalities and our ageing population [15].

Current data suggest that metastases originate from clonal cell subpopulations within genetically and phenotypically heterogeneous primary tumours [16-18]. The physiological requirements can be conceptually summarised in a model comprising initiation (invasion and dissemination via blood/lymphatics), progression (survival, extravasation) and organ colonisation [19]. Colonisation of the brain is a very inefficient process. Cells must actively cross the specialised blood-brain-barrier (BBB) and mouse models suggest there is huge attrition of cells that initially infiltrate this foreign microenvironment [20]. Those that successfully evade local defences adhere to the outsides of capillaries, proliferate to form a micrometastatic sheath and may track along the vessels to facilitate spread [20,21]. Vascular co-option remains important in larger BM, though angiogenesis is also involved in sustaining increasing metabolic requirements, and the relative contributions of these mechanisms may vary according to primary tumour type [22]. The BM microenvironment is complex, comprising neurons and neural progenitor cells, tumour-associated parenchymal cells (e.g. reactive astrocytes, endothelia, microglia), perivascular niches and heterogeneously distributed areas of oedema, hypoxia and necrosis. Several studies have highlighted the role of this unique milieu in driving speciation of cancer cells in the brain [23-26] and in therapeutic resistance [22,27].
BM are not routinely resected and so the availability of clinical samples for research has been limited. Most research has been based on archival formalin-fixed samples, primary tumour datasets with brain relapse data, or experimental models. These approaches have implicated several mechanisms in the development of BM; for example, p53, Wnt/TCF, HB-EGF, COX2, BMP-2, src, serpins and cathepsin-S [21,24,26,28-31]. However, the clinical relevance of many existing candidates is not fully understood. Detailed molecular analysis of human tumours could help address this deficiency, and also highlight opportunities for drug repositioning, identify new targets and improve our understanding of the natural history.

The ERBB/HER pathway has also been implicated in BM development, particularly the tyrosine kinase receptors EGFR, HER2 and HER3 [7,29,32-35]. These receptors engage in ligand-induced dimerization, activating downstream signalling through PI3K/AKT, PLCγ/PKC, STAT and Ras/MAPK pathways. The most potent oncogenic unit is the HER2-HER3 heterodimer [36]. HER2 is an orphan receptor. HER3 has diminished tyrosine kinase activity and is thought to be indispensable in breast tumours driven by ERBB2 amplification [37]. Upon binding extracellular neuregulin ligands (NRG1 or NRG2), HER3 allosterically activates other receptors: mainly HER2, and to a lesser extent EGFR and HER4 [36,38-40]. Recent evidence suggests HER3 may be capable of homodimeric signalling provided there is sufficient HER2 expression to initiate the accumulation of signalling-competent HER3 homodimers [41].

Cancer genome sequencing projects that catalogue genomic alterations are beginning to shed light on mutational processes, identify genomic drivers and demonstrate the full extent of heterogeneity in cancer [42,43]. These data provide a foundation for pharmacogenomics research and precision medicine. Although brain relapse accounts for the highest rates of cancer-related morbidity and mortality and molecular-targeted therapies are critically under-utilised for established BM, very few studies have comprehensively catalogued and analysed metastatic brain tumour genomes.
BM often develop in patients who have received prior treatment for systemic and/or primary
disease (e.g. cytotoxic chemotherapy, molecular-targeted therapy). In order to survey the spectrum
of genomic alterations in these highly refractory tumours despite differences in their genomic and
physiologic evolution, we performed copy-number analysis, RNA and exome sequencing on a
cohort of 36 BM from primary breast cancer, lung cancer, melanoma and oesophageal cancer. We
then analysed the data to investigate aspects of brain metastasis biology and address issues of
clinical relevance: (1) integrated the datasets to identify genes and pathways that were recurrently
or significantly altered across the cohort; (2) explored the diagnostic utility of genomic profiling for
BM of unknown primary origin; (3) investigated actionable biomarkers; and (4) interrogated the
datasets and used immunohistochemistry to investigate the ERBB/HER pathway and the
applicability of anti-HER therapies for treatment of established BM.
Methods

Brain metastases (BM; n=36) were collected and snap-frozen within two hours of collection. Blood samples were taken immediately before surgery. All patients provided informed, written consent to use their samples for this study, which was approved by relevant human research ethics committees.

A detailed description of sequencing, mutation calling, verification and subsequent analyses is included in Supplementary Information. Briefly, BM were macro-dissected on ice, then DNA and RNA were isolated simultaneously. DNA copy-number alterations were identified using SNP arrays (Illumina Human Omni 2.5M) and the GAP tool [44]. Exome sequencing of matched BM and whole blood DNA, and sequencing of BM RNA were performed using an Illumina HiSeq 2000 instrument. Sequence data were mapped to a GRCh37 assembly using BWA [45]. Substitutions were detected using qSNP [46] and GATK [47] and indels called with Pindel [48] (BAM files are available in the EGA; EGAS00001000722). Tumour cellularity was determined using qpure [49]. Non-synonymous mutations were analysed using IntOGen-mutations [50] and MutSig [43] tools. Pathway analysis was conducted using Ingenuity® Pathway Assist.

For the HER/ERBB pathway, formalin-fixed, paraffin-embedded BM (n=167) were sampled in tissue microarrays, then analysed for expression of phosphorylated HER receptors by immunohistochemistry (IHC; see supplementary methods).
Results

*The genomic landscape of human brain metastases.*

Thirty-six fresh-frozen BM (11 breast, 18 lung, six melanoma and one of oesophageal cancer origin; Table-1) were subjected to DNA copy-number analysis, exome- and RNA-sequencing. Globally, copy-number alterations in BM were typical of the respective primary tumour types (Figure-1, Figures S1-3, Tables S1-5) [51-53]. For example, ER+ breast-BM exhibited less complexity than HER2/ER-negative cases [54,55]. Copy-number change in lung-BM was variable, while melanoma-BM genomes were relatively silent in terms of copy-number aberrations. Using the Gistic tool, we identified 339 significantly amplified regions (including *EPCAM, JAK2, HRAS, KRAS* and *FOXA1*) and 59 significantly lost regions, including *NRG1, CDKN1B, TP53 and PTEN* (Figure-S3).

We identified 22,754 somatic single nucleotide variants (SNVs) and small insertion-deletions (indels) (Tables S6-8), of which 16,785 were non-synonymous mutations affecting 8,305 genes [46-48,56]. Somatic mutation burden varies substantially between different cancers. Generally, haematologic and childhood cancers have the lowest mutation rates, whereas those linked to chronic mutagen exposure (e.g. UV-light and smoking) have the highest [42,43]. Consistent with this, melanoma-BM harboured the most coding mutations (average 1,906; range 688-3,283/case), followed by lung-BM (average 491; range 101-921/case) and breast-BM (average 210; range 33-822/case) (Figure-2A, Table-S6).

**Identification of candidate genes associated with brain metastasis**

Known mutated drivers of primary breast, melanoma and lung cancers were frequently mutated in BM. For example, *TP53* was mutated in 25/36 cases and *PIK3CA* was mutated or amplified in 16/36 cases [17,51,52,57,58] (Figure-2A). *KRAS* and *BRAF* were mutated in 11/18 lung-BM and 3/6 melanoma-BM respectively [52,57]. *CDKN2A* (p16) was lost with barely detectable expression (mean 1.79 transcript counts/million (cpm)) in 6/6 melanoma-BM (Figures S4-6, Table-S9). Twenty-six genes were significantly mutated according to IntOGen-mutations analysis [50], which ranks genes according to the predicted functional consequences of mutations they harbour. These
included TP53, KRAS, and DSC2 (p<1E-09, p=2.16E-09, p=3.52E-09 and 2.49E-07, respectively; Table-S10). DSC2 encodes desmocollin 2, a calcium-dependent cadherin superfamily member involved in epithelial desmosome formation. Six of the 26 genes were significantly mutated according to the MutSig tool [43], which predicts significance based on the background mutation rate of each gene: TP53, KRAS, ST7 (tumour suppressor gene [59]), PIK3R1 (PIK3CA regulatory subunit), SMC5 (DNA double-strand break repair [60]) and NRAS (p<0.05).

We also investigated primary cancer type-specific mutations in breast-, lung- and melanoma-BM, using a variety of criteria including mutation frequency, gene size, the ratio of synonymous to non-synonymous mutations and their predicted functional consequences (Table-S11). Some genes were mutated substantially more frequently in BM compared to unmatched primary cancers (TCGA and COSMIC datasets), and therefore may warrant targeted analysis in matched BM and primary tumour samples to investigate clonal enrichment. For example, COL5A1 and MAP3K4 were frequently mutated in breast-BM (68- and 34-fold enrichment, p=7E-05 and 2.39E-04, respectively; Table-S11). The ITPR1 gene was also in this category (17-fold enrichment; 3/3 assessable mutations predicted to be deleterious). Interestingly, ITPR1 expression is inversely associated with metastasis to the brain, but not other sites in breast cancer (HR>4.0, p<0.05; Figure-S8) [29,61]. ITPR1 encodes the intracellular receptor for inositol 1,4,5-trisphosphate and is involved in intracellular calcium signalling including endoplasmic reticulum stress-induced apoptosis. RNAseq analysis showed that mutant allele expression was generally undetectable or very low for all the aforementioned candidate BM genes (Figure-S6; Table-S7).

**Identification of candidate pathways associated with brain metastases**

To identify gene networks that are recurrently altered in BM, we performed canonical pathways analysis of mutated and integrated genomic driver genes (those for which copy-number alteration was correlated with absolute expression across the cohort) (Tables S12-13). Of the 46 pathways over-represented in this dataset, the most significantly altered was ‘axon guidance signalling’, which is involved in actin cytoskeleton reorganisation in response to migratory guidance cues (p=2.19E-05; Table-S12, Figure-2A, Figures S5-6). It includes Slit-Robo, Netrin, Semaphorin and
Ephrin signalling, which have diverse functions during development, in normal tissues and cancer [62-64]. ‘Protein Kinase A signalling’ and ‘role of NFAT in cardiac hypertrophy’ (including calcium-calcineurin signalling-regulated processes) were also significantly altered (p=3.24E-04 and 1.66E-03, respectively). Seventeen networks were overrepresented upon integration with expression data but not when mutated and copy-number-altered genes were analysed independently (Table-S12), suggesting key roles for integrated genomic drivers in these pathways. Interestingly, this included HER2- and GABA-receptor signalling, and DNA double-strand break repair, which have previously been associated with BM development [25,65].

Clinical applications for genomic profiling

BM are not routinely biopsied in current clinical practice, nor are molecular targeted therapies used as standard treatment for established BM. However, moving towards more personalised models of cancer treatment, we will be faced with the challenge of adapting these practices to offer patients the most appropriate and effective care [66,67]. We therefore applied the BM genomic data to explore several clinically relevant scenarios:

Diagnostic utility for cancers of unknown primary (CUP). According to information available at the time of sample collection and sequencing, the primary cancer-of-origin was unknown for Q631 and Q739. Based on the idea that patterns of somatic mutation can mark underlying mutagenic mechanisms in cancer [42,43,52,68,69], we applied mutational signature analysis to predict the primary type for these samples [42]. This revealed signatures associated with smoking, UV exposure, APOBEC activity and ageing (Figure-2B, Figure-S7). Fifty to ninety percent of mutations in lung cases were consistent with the smoking signature, whereas >90% of melanoma-BM SNVs comprised the UV signature. The smoking signature contributed the majority of mutations harboured by Q631 and Q739 (80/73.6%, respectively), suggesting that the primary site-of-origin was lung cancer, which was later confirmed after sourcing additional clinical information (external radiology and treatment data).

Actionable genomic alterations. We investigated mutation and copy-number-driven expression for a panel of predictive genomic markers [67], identifying alterations with published evidence of a
genotype-drug efficacy relationship in 31/36 (86%) cases, including 25 with clinical evidence supporting a genotype-drug efficacy relationship (Table-2). This analysis exemplifies potential therapeutic opportunities identified by genomic profiling of resected BM.

**Limited therapeutic options due to no/limited diagnostic information available for the primary tumour.** Two breast-BM were in this category: for Q751, the patient was treated for primary breast cancer abroad in 1986; and for Q639, the patient presented with lymph node metastases, and despite thorough diagnostic analysis of her bilateral mastectomy tissue, the primary tumour could not be located. This patient was a germline **BRCA1** mutation carrier and the histopathology was consistent with metastatic breast cancer. In both cases, we identified therapeutic opportunities: Q751 harboured an activating mutation in **PIK3CA** (p.H1047R), and Q639 harboured genomic loss/suppression of **PTEN**; both associated with sensitivity to PI3K/AKT/mTOR pathway inhibitors (Table-2).

**Discordance in expression of key prognostic/predictive biomarkers between primary and metastatic disease.** We identified an **ERBB2**-amplified BM (Q851), however the matching primary breast cancer was clinically classified as HER2-negative around four years earlier based on IHC and SISH analysis of a core biopsy (the only diagnostic specimen available as the patient was treated with chemo- and hormone-therapy in the neo-adjuvant setting). The patient had four distant relapses over five years (one liver lesion, three BM), none of which were subjected to diagnostic HER2 testing. Prompted by the serendipitous SNP-array finding, retrospective HER2 IHC and SISH analysis were performed on all five diagnostic specimens, and showed progressive enrichment for **ERBB2** amplification and expression. The patient was subsequently offered HER2-targeted treatment, beginning with lapatinib/capecitabine therapy which had clinical activity against treatment-naïve BM in a phase-II trial [70] (Figure-3). The patient is currently alive and stable with metastatic disease.
**Expression and activation of the actionable HER pathway in brain metastases**

There is a growing body of literature implicating ERBB/HER pathway activation in development of BM [29,32,33,35]. Therefore taking a candidate approach, we analysed mutation, copy-number and expression of key ERBB/HER pathway members (Figure-2A, Figures S5-6 and S9-10).

*PIK3CA* was altered in 17 samples, including five cases with amplification-associated over-expression and three cases with the known hot-spot mutation H1047R (Table-2, Figure-S9).

Consistent with previous reports [71,72], we observed copy-number loss affecting *PTEN* in 5/11 breast-BM (associated with very low expression in the two triple-negative cases; Table-2). Each of the four *ERBB* genes was amplified in at least one case. For *EGFR* and *ERBB2*, copy-number correlated with expression across the cohort (p<0.0001; Figure-S9). *ERBB2* transcripts were abundantly expressed in lung- and breast-BM (outliers by inter-quartile range criteria; Figure-4, Figure-S4A), whereas expression of *EGFR* and *ERBB4* was variable across primary cancer types. Of the four receptors, *ERBB3* was the most highly and consistently expressed across different primary tumour types (Figure-4A), and whilst its expression was not associated with copy-number (Figure-S9), *ERBB3* transcript abundance correlated with its oncogenic partner *ERBB2*, even in non-*ERBB2*-amplified tumours (p<0.0001; Figure-4B).

Regarding the receptor ligands, we observed recurrent loss at 8p12 affecting the HER3 ligand *NRG1* in 7/11 breast-BM, including all five *ERBB2*-amplified cases (Figure-2A). Irrespective of copy-number, expression of *NRG1* and *NRG2* was very low across the cohort (<1.0 cpm in 9/32 cases; Figure-4C), suggesting that HER3-positive tumour cells are more likely to source ligand from the neuregulin-rich brain microenvironment [73] than from within the tumour itself. In contrast, *EGFR* and HER4 ligands were variably expressed, generally highest in lung-BM (Figure-S10).

To investigate how these findings relate to pathway activation, we used immunohistochemistry (IHC) to analyse phosphorylated HER isoforms in an independent cohort of 167 archival BM originating from breast, melanoma, lung, colorectal, prostate, ovarian and renal cancers. Overall, the most ubiquitously activated receptors were HER3 and HER4, with strong complete membrane staining in 57.7% and 52.6% cases respectively (Figure-5, Figures S11-12). Since HER3 activity
can be inhibited indirectly with clinically approved HER2-targeted agents (e.g. lapatinib, trastuzumab, pertuzumab), we determined the HER2 status of the archival BM according to clinical diagnostic criteria (Figures S11-12). 76.7% of HER2-positive cases showed strong HER3 activation, though HER3 was also frequently activated in HER2-negative cases (only 34.7% of the HER3-activated cases were HER2-positive; Figure-5C). 82.1% of HER3-activated cases showed some degree of HER2 activation. Collectively these data provide support to the applicability of HER2/3-targeted therapies for management of BM.
Discussion

A diagnosis of metastatic brain disease marks a serious physiological and psychological downturn for cancer patients. The current mainstays of treatment are whole-brain or stereotactic radiotherapy and/or surgical excision. After treatment the outcome remains poor, highlighting an urgent need for new therapeutic options. Clinical data on the efficacy of targeted therapies against established BM is limited due to an historic lack of prospective studies in this setting, although this trend is beginning to change, with several agents now being assessed in phase I/II clinical trials [22,70,74]. And so, notwithstanding the challenges (e.g. variability in intratumoural perfusion and drug uptake, development of resistance [1,75,76]), pre-clinical and early clinical studies with targeted agents are providing an optimistic outlook [22]. Diagnostic genomic profiling of resected BM could be used to rationalise the selection of patients for clinical trials and identify new treatment options for patients in the future.

This is the first study to apply genomic and transcriptomic profiling to a cohort of BM. Globally, they were more similar than different to the respective primary tumour types, consistent with previous reports [16,17,77-80]. However, differences were also identified that could provide insight into BM development with further analysis. BM are complex tumours with diverse aetiologies, and so tumour cell-intrinsic mechanisms contributing to their growth are likely to be highly variable. Conversely, studies demonstrating BM exploitation of neural growth pathways [23-25,76,81] raise the possibility that convergence of diverse metastatic entities in common soil may underpin recurrent, targetable features [22]. We explored this by interrogating genomic and transcriptomic data from BM of different origins. As the cohort sizes were limited, we applied multiple filters to prioritise genes and pathways of interest. Analysis of mutation significance and frequency highlighted new candidates, including DSC2, ST7, PIK3R1, SMC5, COL5A1, MAP3K4 and ITPR1. The repertoire of alterations was enriched with functional gene networks, with axon guidance, protein kinase A and calcium signalling-related processes amongst the most significant. While further investigation is required to establish whether these candidates are involved in BM development, it is encouraging that pathways previously implicated in BM were also identified in this study (e.g. HER2, GABA-receptor signalling and DNA double-strand break repair [25,65]).
Targeted analysis of actionable genomic alterations identified potential drug targets in 31/36 cases, including 25 with clinical evidence supporting a genotype-drug efficacy relationship (Table-2). This was a retrospective study and it is uncertain if the genomic data would have led to deployment of alternative therapeutic agents if known at the time of BM resection, but it is possible this information may have impacted on clinical management in some cases (e.g. clinical trial eligibility). For example, 10 patients in this study were eligible for a phase-I trametinib (MEK-inhibitor) trial for patients with established BM from NRAS- or KRAS-mutant cancers [74].

We had the opportunity to translate a serendipitous finding to therapeutic intervention for one breast cancer patient, who was still alive at completion of the study despite multiple recurrent BM. This case exemplified several challenges in the management of metastatic brain disease that are underpinned by intratumoural heterogeneity: 1) limited tumour sampling for diagnostic analysis (e.g. core biopsy); and 2) predictive biomarker discordance between primary and metastatic disease. In this case there was progressive enrichment of HER2-positivity in four distant metastases over five years. We hypothesise that the bulk of the primary tumour and disseminated cells at that stage (ER/PR-positive, HER2-negative) responded to hormone- and chemotherapy, leading to outgrowth of ER/PR-negative, HER2-positive subclone(s). Indeed, others have documented an association between HER2 discordance in BM and hormone therapy for primary breast cancer [82]. This finding supports the ASCO/CAP recommendation to perform HER2 testing of metastatic breast cancer (particularly where the primary was HER2-negative) if all patients who may benefit from targeted therapy are to be offered an opportunity [83,84]. We concur with some authors, who have suggested inclusion of the presence of HER2 intratumoural heterogeneity in routine breast histopathology reporting [85-87].

There have been a number of therapeutic developments targeting the HER family; for example, erlotinib and gefitinib, which target HER1/EGFR; trastuzumab, pertuzumab and T-DM1 (HER2); patritumab and MM-121 (HER3); and multi/pan-HER inhibitors such as lapatinib, MM-111, afatinib and dacomitinib. The extent to which HER receptors are activated in different types of BM has not been characterised, yet this is a critical step if HER-targeted therapies are to be considered for
clinical management. Here, we showed that both HER3 and HER4 are extensively activated in BM. The roles of HER4 isoforms are complex and context-dependent [88-92]. The abundance of membranous phospho-HER4 in human BM warrants more detailed analysis of HER4 isoforms in relevant experimental models. HER3 has been implicated in microenvironment-driven tumour growth [32,33,93], brain microvascular permeabilisation [34] and therapeutic resistance [37,94-98]. Its oncogenic activity has been characterised mainly in the context of HER2-addicted cancers as the pair are regarded as obligate partners. However, recent data suggests that HER2 transactivation of HER3 is not dependent on equal surface levels of these receptors and that HER3 homodimers are ~three-fold more stable than HER2-3 heterodimers [41]. We found that only 34.7% of pHER3-positive BM were HER2-positive by clinical diagnostic criteria, but more than 80% expressed pHER2 to some degree. Therefore targeting HER3 directly may be a more broadly applicable strategy than indirect targeting via HER2 dimerization inhibitors.

It is also important to consider the IHC data in the context of HER2-positive breast cancer. Around 50% of these patients develop BM over time [99,100]. The fact that adjuvant trastuzumab treatment for primary disease does not alter the overall incidence of BM has contributed to a view that the BBB prevents uptake to efficacious levels, creating a sanctuary site for metastatic cells. However, this remains to be equivocally demonstrated in human subjects and there are several lines of evidence suggesting that inefficient delivery is not solely responsible. Firstly, the presence of BM is often an exclusion criterion in clinical trials in the primary disease setting, as patient prognosis is deemed incompatible with many trial endpoints. Where patients with BM are included but there is minimal or no impact on intracranial disease progression, it is not possible to accurately interpret efficacy against established BM because dose selection is critical for achieving optimal tumour concentration [101], and evidence suggests the BM microenvironment impacts significantly on uptake of circulating drugs [1,102,103]. Second, several PET imaging studies have now demonstrated accumulation of trastuzumab in HER2-positive BM [104-106]. Finally, others have recently demonstrated uptake of T-DM1 and bevacizumab (anti-VEGFR) to therapeutic levels in BM [22]. These data highlight promising opportunities with mAbs and mAb conjugates (e.g. T-
DM1, radioimmunotherapy), and that it could be time to rethink the perceived limitation imposed by the BBB on treatment of established BM [22].

We postulate that the neuregulin-rich brain microenvironment also contributes to therapeutic resistance in BM. HER2 and HER3 are induced in BM from breast and lung cancers [32,33,35], and our data suggest this is an adaptive response to paracrine ligand (Figure-S13) since most BM lacked expression of NRG RNA. Neuregulin-1 mediates resistance to cisplatin therapy in mouse models of non-small cell lung cancer [107] and this may also be relevant in BM, which are essentially treatment-refractory manifestations of cancer arising in a neuregulin-rich microenvironment. HER3 is causally associated with trastuzumab escape in primary breast cancer [108] and in our cohort was activated in 55% of HER2-positive breast-BM. The incidence of BM in HER2-positive breast cancer patients treated with trastuzumab and docetaxel, with/without pertuzumab (a HER2-HER3 dimerization blocker) was recently evaluated as part of the CLEOPATRA study [109]. In patients with controlled extracranial disease where the brain was the first site of relapse, pertuzumab significantly delayed the onset of BM and though the cohorts were small, survival also showed an encouraging trend. These data suggest that comprehensive mechanistic blockade of the HER2-HER3 dimer could be beneficial for patients with established BM from HER2-positive breast cancer.

To our knowledge, this is the first study to characterise the genomic landscapes of BM. The data revealed novel candidates, potential clinical applications for genomic profiling of resectable BM, and highlight the possibility of therapeutically targeting HER3, which is broadly over-expressed and activated in BM independent of primary site and systemic therapy.
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This work was supported by funding from the Australian National Health and Medical Research Council (APP1030751) and The National Breast Cancer Foundation, Australia (fellowship to PTS). We would like to thank D. Gwynne for central co-ordination at the Queensland Centre for Medical Genomics and Professor Sir Mike Stratton for his support in attracting funding for this study. We wish to thank Heather Thorne, Eveline Niedermayr and all the kConFab research nurses and staff, and the many families who contribute to kConFab. We would also like to acknowledge the Brisbane Breast Bank for coordinating sample collection, archiving and data management, as well as all the patients who donated tissue for this study.

Authors’ contributions


Table-1. Basic clinical information for the sample cohort.

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<th>Sample ID</th>
<th>Primary type</th>
<th>ER</th>
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<th>Histological type</th>
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<th>Progression (years)</th>
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<td>46.88</td>
<td>49.51</td>
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</table>

Footnotes:
* Biomarker status for breast samples was obtained from clinical diagnostic pathology reports; ER status was assessed by immunohistochemistry (+, ≥1% tumour cells stained); HER2 status determined by chromogenic in situ hybridisation (CISH).
+ Cellularity was determined using the qPUR tool [49]
Histological type derived from the metastatic brain lesion (all others from primary tumour analysis)
Samples harbour pathogenic germline BRCA1 mutations: Q639 (BRCA1c.4327 C>T (p.Arg1443X)) and Q851 (BRCA1c.5278-del (exon21_24del))

Abbreviations: +, positive; -, negative; BM, brain metastasis; Adeno, adenocarcinoma; Adeno-squam, mixed adeno-squamous carcinoma; ER, oestrogen receptor; HER2, human epidermal growth factor receptor 2; IDC, invasive ductal carcinoma; LCC, large cell carcinoma; NA, not available; ND, not determined; P, primary; SCC, squamous cell carcinoma; surg, surgery.

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### Table-2: Potentially actionable genomic alterations in brain metastases.

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<tr>
<th>Gene</th>
<th>No. cases</th>
<th>Alteration</th>
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<th>Lung-BM</th>
<th>Mel-BM</th>
<th>Oes-BM</th>
<th>Drugs</th>
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<td>MEK/PI3K/mTORi&lt;sup&gt;ii&lt;/sup&gt;</td>
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<td></td>
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<td>p.G12V</td>
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<td>Q631 (68.32), Q782 (79.59)</td>
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<td>MEKi&lt;sup&gt;i&lt;/sup&gt;, PI3K/mTORi&lt;sup&gt;ii&lt;/sup&gt; [110,111]</td>
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</table>

**Footnotes**

Analysis of these actionable genes is intended to exemplify possible opportunities for genotype-directed therapy, but is not exhaustive. A three-category system was used to classify alterations based on the evidence linking genotypes to drug efficacy [118]:

- **a** Strong clinical evidence supporting the efficacy of a drug based on tumour genotype.
- **b** Compelling pre-clinical evidence for efficacy of a drug based on tumour genotype.
- **c** Where a drug could be deployed based on a known gene-drug relationship, but where there is currently limited or inconclusive evidence supporting the efficacy of that drug in the context of the observed tumour genotype.

For copy-number alterations, only those associated with corresponding over-/under-expression were included (expression in 1st/4th quartiles for CN loss/gain, respectively, in BM of the same primary type, and/or <2 counts/million. * denotes homozygous loss. **For amplified genes in Q734, over-expression was defined as nCPM in the 4th quartile of expression for all other primary types. For Q452, RNAseq data was not available but ERBB2 amplification was retained as this currently qualifies cancer patients for HER2-targeted therapy. Variant allele expression frequency is indicated in parentheses for actionable hotspot mutations.**

Abbreviations: Amp, genomic amplification; CN, copy-number; mel, melanoma; oes, oesophageal.
**Figure legends**

**Figure-1. Copy-number landscape of brain metastases.** The heat map summarises GISTIC 2.0 analysis of copy-number losses (blue), gains (red) and diploid states (white); colour intensity is proportional to peak amplitude (Tables S2-5). Samples are grouped according to primary cancer type (breast, lung, melanoma and oesophageal (E)). *Lower panel:* sample codes and ER/HER2 status for breast-BM (black fill, positive; grey fill, negative; no fill, information not available; P, primary tumour; BM, brain metastasis. Data taken from clinical diagnostic pathology reports for primary breast tumours. For BM, ER status was from clinical reports and HER2 status was extracted from SNP-array data (GAP score ≥ 6). All breast cases were invasive ductal carcinomas (IDC). *Q639 and Q851 harboured germline BRCA1 mutations (Table-1).
Figure-2. (A) Mutation frequencies and integrated genomic analysis of key genes in brain metastases. Upper panel: overall mutation frequencies for the 36 BM (single nucleotide variants (SNVs) and small insertion/deletions (indels)). The matrix shows SNVs, indels and copy-number alterations affecting key cancer genes, novel candidate genes (Tables S10-11), core members of the Axon guidance and ERBB/HER signalling pathways. The histogram on the right shows the proportions of different alterations and the total numbers of samples harbouring alterations in each gene. (B) Mutational signatures in human brain metastases can be indicative of primary cancer of origin. The histograms display SNV transition/transversion rates (upper), and relative proportions
of four mutational signatures identified in the dataset (lower). For Q739 and Q631 (red), primary cancer types were unknown at the time of sample collection/sequencing. They were predicted to be lung cancer-derived based on smoking signature-dominated mutation profiles, confirmed by sourcing additional clinical information. *Germline BRCA1 mutation. Lower panels denote ER/HER2 status for primary breast (P) and metastatic brain (BM) tumours.

**Figure-3.** Case demonstrating enrichment for HER2 expression and selection of *ERBB2*-amplified clone(s) in brain metastases. SNP-array analysis identified discordant HER2 status for sample Q851, an *ERBB2*-amplified BM that originated from HER2-negative breast cancer. The
patient had four distant relapses over five years (one liver lesion and three BM). Retrospective immunohistochemical (IHC) and silver in situ hybridisation (SISH) analysis of all diagnostic specimens revealed progressive enrichment for HER2 expression in the metastatic deposits, with a corresponding increase in the average number of ERBB2 copies/cell over time. The figure shows the relevant clinical history, retrospective diagnostic (dx) HER2 data and representative HER2 IHC staining for the five samples. FEC, fluorouracil (5FU) + epirubicin + cyclophosphamide chemotherapy regimen; L, left; R, right; WBRT, whole-brain radiotherapy. Scale bar = 0.5 mm.

**Figure-4.** Expression of ERBB receptors and HER3 ligands in brain metastases. (A) ERBB absolute expression (normalised counts per million, nCPM) for each sample. ERBB3 was expressed in the top 95th percentile of all genes for breast-, melanoma- and oesophageal-BM, and was also highly expressed in lung-BM (outliers by inter-quartile range criteria; Figure-S4A). (B) Absolute levels of ERBB2 and ERBB3 transcripts were significantly associated by both linear regression ($r^2=0.4955$, $p<0.0001$) and Spearman correlation analysis ($r=0.432$, $p=0.014$). (C) nCPM values for the HER3 ligands NRG1 and NRG2. Boxes and whiskers show the median, quartiles and the range of expression respectively.
**Figure-5.** Analysis of HER activation in brain metastases from different primary cancers-of-origin. Tissue microarrays containing BM samples (n=167 in duplicate) were stained with specific antibodies targeting pEGFR (Y1068), pHER2 (Y1221/1222), pHER3 (Y1222) and pHER4 (Y1162). (A) Representative staining of the antigens indicated (primary tumour type and score in parentheses); bv, blood vessel; n, necrosis. (B) Overall phospho-HER activation frequencies. (C) Case-by-case summary of HER activation according to origin of primary cancer. Strongly membrane-positive cases (score 3-4) are indicated with purple tiles. HER2 status was also assessed by IHC using diagnostic criteria (HER2dx) with HER2-positive cases shown in dark blue. Light blue tiles indicate cases with any phospho-HER2-positivity (>0). The number (n) of BM analysed is indicated for each primary cancer type; Colo, colorectal; O, ovarian; P, prostate.