Genomic Characterization of Brain Metastases Reveals Branched Evolution and Potential Therapeutic Targets

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Brain metastases are associated with a dismal prognosis. Whether brain metastases harbor distinct genetic alterations beyond those observed in primary tumors is unknown. We performed whole-exome sequencing of 86 matched brain metastases, primary tumors, and normal tissue. In all clonally related cancer samples, we observed branched evolution, where all metastatic and primary sites shared a common ancestor yet continued to evolve independently. In 53% of cases, we found potentially clinically informative alterations in the brain metastases not detected in the matched primary-tumor sample. In contrast, spatially and temporally separated brain metastasis sites were genetically homogenous. Distal extracranial and regional lymph node metastases were highly divergent from brain metastases. We detected alterations associated with sensitivity to PI3K/AKT/mTOR, CDK, and HER2/EGFR inhibitors in the brain metastases. Genomic analysis of brain metastases provides an opportunity to identify potentially clinically informative alterations not detected in clinically sampled primary tumors, regional lymph nodes, or extracranial metastases.

SIGNIFICANCE: Decisions for individualized therapies in patients with brain metastasis are often made from primary-tumor biopsies. We demonstrate that clinically actionable alterations present in brain metastases are frequently not detected in primary biopsies, suggesting that sequencing of primary biopsies alone may miss a substantial number of opportunities for targeted therapy. Cancer Discov; 5(11); 1–13. ©2015 AACR.
Genomics of Brain Metastases

INTRODUCTION

Brain metastases, most frequently originating from melanoma and carcinomas of the lung and breast, are the most common tumor in the brain. Approximately 200,000 cases are diagnosed annually in the United States alone. Patients frequently develop brain metastases even while their extracranial disease remains under control (1). Median survival ranges from 3 to 27 months following metastatic spread to the brain (1). Of patients who have clinically symptomatic brain metastases, approximately half succumb to the cancer in their brain (2). Unfortunately, treatment options are limited, and most current clinical trials in the United States exclude patients with brain metastases.

Because cancers are genetically heterogeneous (3–9), sampling a cancer in two different locations is expected to reveal mutations exclusive to each sample. Furthermore, because brain metastases are often resected during clinical care, such tissue provides an immediate opportunity for genomic assessment of these life-threatening lesions. To date, the extent to which brain metastases, often manifesting years after the primary malignancy, share the genetic profile of the primary tumor remains unknown. Massively parallel sequencing of brain metastases has been performed on a limited number of cases (7, 10), showing novel alterations in the metastatic site. Prior studies have suggested activation of the PI3K pathway in brain metastases (11, 12). Some gene expression signatures have been associated with metastasis to the brain (13, 14).

We performed whole-exome sequencing on 86 “trios” of patient-matched brain metastases, primary tumors, and normal samples, all of which were collected in the course of clinical care (e.g., for diagnosis, symptom control, or restaging). For 15 patients, we also characterized multiple metastatic brain lesions, distal extracranial metastases, and additional samples from the primary tumor or associated regional lymph nodes. Our objectives were to (i) determine whether clinically sampled brain metastases harbor distinct potentially clinically informative mutations not detected in paired primary-tumor samples; (ii) determine the extent to which such mutations are shared among multiple regions of a single brain metastasis, anatomically distinct brain metastasis sites, and temporally separated lesions (in cases that recurred following therapy); and (iii) determine whether lymph nodes or extracranial metastases are genetically similar to brain metastases and might serve as their proxy for genomic assessment and clinical decision making.

RESULTS

Patients

Clinical characteristics of the 86-patient case series are shown in Supplementary Table S1. The majority of the cases were derived from lung (n = 38), breast (n = 21), and renal cell carcinomas (n = 10). Of the 86 patients, 48 had a single brain metastasis, whereas the rest of the cases had additional brain metastases diagnosed radiographically.

Genetic Divergence of Brain Metastases and Primary Tumors

Several lines of evidence indicate that tumors exhibit genetic heterogeneity both across different anatomic regions (3–6, 8, 15) and within single cancer-tissue samples (7, 9, 16, 17). We applied previously described computational methods to address the heterogeneity of cancer-tissue samples and inferred the evolutionary relationship between the sequenced tissue samples from each patient (16, 18–20). We integrated data from somatic point mutations and copy-number alterations to estimate the fraction of cancer cells harboring each point mutation; that is, their cancer-cell fraction (CCF; refs. 16, 18–21). Analysis of the CCF for each mutation across the tissue samples derived from the same patient allowed us to infer phylogenetic trees relating all cancer subclones detected (Supplementary Figs. S1–S6).

Corroborating prior observations, all clonally related primary tumor and brain metastasis samples were consistent with a branched evolution pattern (4, 22). Although they shared a common ancestor, both the primary tumor and the metastasis continued to evolve separately, reflected by (i) the presence of distinct mutations (“private mutations”) with a CCF = 1 (i.e., present in all cancer cells) in both samples (Supplementary Figs. S1 and S7); and (ii) the fact that each sample continued to develop minor cancer-cell populations defined by mutations with CCF < 1.

We failed to identify a minor cancer-cell population in any primary-tumor sample that was the ancestor of its paired metastasis. Such a metastasis-founding subclone would harbor mutations in a subset of the cancer cells of the primary-tumor sample (CCFprimary < 1) that were present in all cancer cells (CCFmet = 1) of the metastasis sample (Supplementary Fig. S7B). Although it is possible that more comprehensive sampling of primary-tumor tissue might have revealed such founding ancestor subclones (20, 22), this would not have been clinically feasible in most cases.

In four of 86 primary/metastasis pairs analyzed, we did not identify common mutations between the primary tumor and metastasis samples, suggesting that they were clonally unrelated (Supplementary Fig. S7C). Three of these arose in the lungs of smokers, with multiple histologically distinct primary tumors diagnosed clinically. An additional patient with breast cancer had another primary tumor in the contralateral breast; this patient was found to harbor a heterozygous germline BRCA1 (5385insC) allele. These 4 patients likely developed...
multiple clonally independent cancers in the context of exposure to tobacco carcinogens or germline risk, suggesting that their brain metastases arose from separate primary tumors (unavailable for analysis).

In many cases, we identified potentially clinically relevant mutations in the brain metastasis that were not detected in the clinically sampled primary tumor. Because the primary and metastatic tissue samples were fully diverged siblings with no detectable overlap of subclones, we calculated power to have observed these mutations in the primary-tumor samples assuming a CCF of 1.0. However, it could be argued that small subclones representing ancestors of the metastasis might have been present in the primary samples, but not detected (because their CCF would not significantly displace that of their sibling subclones with apparent CCF = 1.0 in the primary sample). We therefore also calculated the minimum CCF of these mutations in the primary sample for which we had detection power ≥ 0.95 (minimum CCF95).

For example, in a patient who had undergone resection of a primary renal cell carcinoma (case 218), but subsequently developed both extracranial metastases 3 years after resection and a brain metastasis 7 months later while on bevacizumab for progressive extracranial disease, we detected a homozygous PTEN nonsense mutation in the brain metastasis, but not in the primary-tumor sample. Biallelic loss of PTEN may correlate with sensitivity to some PI3K/akt/mTOR inhibitors (23), and has also been found to mediate resistance to other inhibitors, including EGFR (24) and PI3K inhibitors (25). Deep sequencing of the primary-tumor sample using an independent library further supported the absence of the mutation (0/263 reads; power > 0.99; minimum CCF95 = 0.032). As previously reported in non–central nervous system metastases of clear-cell renal cell carcinoma (ccRCC; ref. 4), we also observed convergent evolution in this case, with distinct PBRM1 frameshift mutations present in the brain metastasis and primary tumor, confirmed with deep sequencing of the primary tumor (Fig. 1A and Supplementary Table S2).

A second patient (24) with a single synchronous brain metastasis from ccRCC had mutations in MTOR, VHL, and PBRM1 that were shared by the metastasis and primary tumor. Additional alterations in PIK3CA (p.E542K) and CDKN2A (homozygous deletion) were detected only in the brain-metastasis sample (Fig. 1B and Supplementary Fig. S8). Deep sequencing with an independent library failed to detect PIK3CA (Supplementary Table S2) in the primary-tumor sample (0 of 733 reads, power > 0.99; minimum CCF95 = 0.014).

A third patient (135) with HER2-amplified breast cancer and stable extracranial disease developed a brain metastasis after 3 years of trastuzumab therapy. The brain metastasis and primary tumor shared an amplification in ERBB2 and a homozygous deletion of TP53; however, the primary tumor harbored an additional MYC amplification that was not observed in the brain-metastasis sample, and the brain metastasis harbored a homozygous missense mutation of uncertain significance in BRCA2 (p.H2563N) that was not detected in the primary-tumor sample (0/82 reads; Fig. 1C). Deep sequencing of an independent library from the primary-tumor sample (0/133 reads; power > 0.99; minimum CCF95 = 0.027) also failed to detect the BRCA2 mutation (Supplementary Table S2).

A fourth patient (0244) with HER2-amplified breast cancer developed a brain metastasis after 2 years of trastuzumab therapy. We detected both a broad amplification (six copies) and an activating point mutation in EGFR (L858R; 7/129 reads) in the metastasis sample. In this case, the mutant L858R allele was not amplified, consistent with the amplification having occurred prior to the mutation (Fig. 1D). Both the amplification and the mutation were not observed in the primary-tumor sample (0/204 reads; Fig. 1D) validated with additional deep sequencing (0/419 reads; power > 0.99, minimum CCF = 0.067; Supplementary Table S2). Although the L858R mutation is common in lung cancers and is associated with sensitivity to gefitinib (26), one proposed mechanism of resistance in anti-HER2 therapy in breast cancer is activation of EGFR (27, 28), suggesting that trastuzumab therapy may have selected for this mutant allele. We also detected an FGFR1 amplification in the brain metastasis and a CCND2 amplification in the primary tumor (Fig. 1D).

A fifth patient (331) with serous ovarian cancer experienced a complete remission for 1 year following chemotherapy and subsequently developed a solitary brain metastasis 5 years later. The brain metastasis harbored a high-level amplification of ERBB2 (32 copies). Using immunohistochemical staining, we confirmed that HER2 was indeed overexpressed in the metastasis and was not detected in the primary-tumor sample (Fig. 1E). Although HER2 amplifications are not commonly observed in serous ovarian cancer (29), such amplification events have been shown to confer sensitivity to anti-HER2 therapy in breast and other cancers (30). We also identified a BRAF amplification in the primary tumor that was not present in the brain-metastasis sample (Fig. 1E). Further amplifications of FGFR1 and MYC were detected only in the brain metastasis (6 and 7 copies, respectively; Supplementary Fig. S9). These five examples demonstrate that genomic sampling of resected brain metastases revealed potentially actionable mutations not detected in the clinically sampled primary tumors.

The Landscape of Clinically Informative Driver Alterations in Clinically Sampled Brain Metastases and Primary Tumors

The genetic divergence observed between clinically sampled primary tumors and brain metastases implies that potentially clinically actionable targets present in the brain metastasis may not be detected from analysis of a single sample of the primary tumor (Fig. 1 and Supplementary Fig. S1). We therefore evaluated the extent to which primary-tumor biopsies and resected brain metastases, collected as part of clinical care, would allow identification of oncogenic alterations with potential clinical significance across our entire series of 86 paired cases. To systematically perform this evaluation, we used the TARGET database (31) of genes for which somatic alterations have therapeutic or prognostic implications (Supplementary Table S3). Many of the TARGET alterations serve as eligibility criteria in the context of genomically guided clinical trials in cancer, both histology specific or independent of histology (31). Alterations in TARGET genes were...
Figure 1. Brain metastases harbor clinically actionable mutations not detected in primary-tumor samples. A–E, phylogenetic trees inferred for five example cases. Branch colors indicate the types of tissue samples descended from each branch (gray, shared by all samples; blue, primary-tumor sample; red, brain metastasis). Darker-colored lines correspond to subpopulations of cancer cells detected with CCF < 1; the maximally branching evolutionary relationships of these clusters are drawn on the ends of each sample branch, surrounded by shaded ellipses denoting the tissue sample. The thickness of each branch is proportional to the CCF of mutations on that branch. Potentially clinically informative (TARGET) alterations (black) and additional likely oncogenic alterations (gray) are annotated onto the phylogenetic branches on which they occurred. Timelines depict the sequence of diagnosis, treatment, and tissue sampling for each case, with chemotherapy treatment intervals denoted by gray rectangles, and treatment with specified targeted agents denoted by orange rectangles. Colored vertical lines denote collection of sequenced cancer tissues (blue, primary; red, brain metastasis). BEV, bevacizumab; BM, brain metastasis; BM1, brain metastasis from one anatomic location; BM2, brain metastasis from second anatomic location; Bx, biopsy; C, chemotherapy; CET, cetuximab; CR, complete response; Dx, diagnosis; EM, extracranial metastasis; I-131, radioactive iodine; LAP, lapatinib; LN, lymph node; PARPi, PARP inhibitor; PBM, progressive brain metastasis; PED, progressive extracranial disease; PI3Ki, PI3K inhibitor; SED, stable extracranial disease; Sx, surgery; SUN, sunitinib; TRA, trastuzumab; WBRT, whole brain radiotherapy; XRT, radiation. E, also shows immunohistochemical staining (IHC) for HER2 in samples of the primary tumor (left), and brain metastasis (right). In addition, genomic copy ratios on chromosome 17 are shown (bottom) for the primary tumor sample (top) and brain metastasis (bottom). Large diamonds correspond to exons of ERBB2, colored according to amplification status (black, unamplified; red, amplified).
Figure 2. The landscape of potentially clinically actionable alterations in brain metastases and primary-tumor samples. A–D, alterations in genes (rows) that may predict sensitivity to the indicated class of targeted agent. Vertical columns correspond to cases, which are ordered by primary histology and presence/absence of alterations. Stacked bar graphs indicating the number of somatic point mutations detected in each phylogenetic branch of each case (columns) are shown at the top of each panel. HER2 status determined during clinical evaluation is denoted by: black, positive; gray, negative; white, not measured. COSMIC, Catalogue of Somatic Mutations in Cancer.
PI3K-mTOR pathway detected only in the brain-metastasis samples. In addition to the PTEN mutation described above (Fig. 1A), another case had a shared small in-frame deletion (p.D52del) in PTEN with an additional splice site mutation detected only in the brain-metastasis sample. A third ccRCC case harbored a PIK3CA E542K mutation (Fig. 1B and Fig. 2B), and a fourth harbored a PIK3R1 N564D mutation previously reported in glioblastoma (36) that activates the PI3K-AKT pathway (37). A fifth ccRCC brain metastasis harbored a small frameshift deletion in PTEN (K66fs) that was predicted to be heterozygous (not shown). Activation of the PI3K-mTOR pathway has been reported in metastatic ccRCC lesions in extracranial sites (4).

We also found mutations that predict sensitivity to HER2/EGFR inhibitors (e.g., trastuzumab, geftinib, cetuximab, erlotinib, lapatinib) in 26 cases in two of four evaluated genes (32 alterations, 20 shared, 2 only in primary-tumor samples, 10 only in brain-metastasis samples). Thirteen of 21 breast cancers harbored amplifications in ERBB2, all of which were shared. In one case (076), we detected an additional activating ERBB2 missense mutation (N777L; ref. 38) only in the brain-metastasis sample in addition to the shared ERBB2 amplification. Notably, 2 patients with lung cancer (Fig. 2C) and a third with ovarian cancer (Fig. 1E) had ERBB2 amplifications detected only in the brain-metastasis samples. Two patients with HER2-amplified breast cancer harbored EGFR alterations detected only in the brain-metastasis samples; in addition to the case above (Fig. 1E), a second patient harbored broad amplification of EGFR (seven copies; Fig. 2C).

The MAPK pathway inhibitor family includes agents that inhibit BRAF and MEK, such as vemurafenib, dabrafenib, or trametinib (31). Thirty-six alterations associated with response to these agents were detected in 29 cases, in 6 of 11 evaluated genes (24 shared, 6 only in the primary samples, 6 only in the brain-metastasis samples; Fig. 2D). Activating mutations in KRAS, which have been associated with tumor responses to MEK inhibitors (39, 40), were the most frequent alteration in this group (19 cases) and were shared in all clonally related cases.

Additional alterations under investigation for association with various targeted therapies, including Ephrin inhibitors, epigenetic therapy, Notch inhibitors, WNT inhibitors, AURKA inhibitors, multia-targeted tyrosine kinase inhibitors, MDM inhibitors, PARP inhibitors, as well as alterations that might be diagnostic or prognostic, are shown in Supplementary Fig. S10.

**Genetic Homogeneity of Brain Metastases**

The discrepancy in the oncogenic alterations detected in clinically obtained samples from the primary tumors and matched brain metastases raised the possibility that every distinct brain metastasis lesion might harbor a unique set of oncogenic alterations. Therefore, we sought to evaluate the extent to which clinical sampling of a single brain metastasis region might be representative of the genetic alterations detected across various sites of intracranial metastasis (Fig. 3A–G). We assessed intraleision heterogeneity (by sampling multiple regions of single brain metastases), as well as interlesion heterogeneity (by sampling from multiple anatomically and temporally distinct brain metastases in the same patient). In each scenario, we observed that all profiled brain-metastasis samples shared mutations that were not detected in the clinically sampled primary tumor, indicating that the subclones sampled in these lesions were more related to one another than to those detected in the primary-tumor sample (Fig. 3A–G). Most importantly, the brain metastases shared nearly all of the potentially clinically informative driver alterations (29 of 30 alterations in 7 samples; Fig. 3A–G).

For four cases (Fig. 3A–C, and G; 0302, 0308, 0314, 0137), we analyzed multiple regions of the same brain metastasis resection. In one example case (0314), we sampled four distinct regions of a cerebellar metastasis from a patient with metastatic HER2-amplified breast cancer (Fig. 3C; 314) and found that each of these metastatic sites shared a PIK3CA mutation (E542K) and an amplification of ERBB2 with the primary tumor. In addition, we found CCNE1 and EGFR amplifications in all of the metastatic brain lesions that were not detected in the primary-tumor sample (Supplementary Figs. S12 and S13). The patient ultimately received treatment with a PI3K inhibitor, with no evidence of intracranial disease progression for 8 months.

For four cases (Fig. 3B, D, E, and G; 0308, 0098, 0176, 0137), we obtained and analyzed samples from brain metastases taken prior to treatment and again at the time of recurrence. For example, in a patient with a large cell neuroendocrine lung cancer (0308; Fig. 3B), we sequenced resections of brain metastases before and following whole-brain radiation and found that each sample shared a MYC amplification (six copies) that was not detected in the primary-tumor sample (Fig. 3B and Supplementary Fig. S14). In another example, a patient with an estrogen receptor–, progesterone receptor–, and HER2-negative (triple-negative) breast cancer (Fig. 3C; 0176) underwent a resection for a symptomatic cerebellar metastasis, and 2 months later had a rapid local recurrence, necessitating resection (Fig. 3E). The primary tumor and brain metastases shared alterations in TP53, PTEN, and MYC. The primary tumor harbored an MCL1 amplification that was not detected in the brain-metastasis samples. We also identified an additional mutation in EZH2 (p.N640S; refs. 31, 41) in both brain metastases but failed to detect this mutation in the primary-tumor sample.

In two cases where anatomically distinct brain metastases were resected, we found that they were closely related to one another and harbored identical potentially clinically informative alterations (Fig. 3F and G). For example, a patient with a HER2-amplified salivary gland ductal carcinoma (Fig. 3F; 0138) developed brain metastases while being treated with trastuzumab. Analysis of a resected approximately 2 cm³ cerebellar metastasis revealed potentially clinically informative amplifications, including MET, CDK6, CCNE1, MYC, and AKT2, that were not identified in the primary-tumor sample (Supplementary Figs. S15–S17). Ten months later, following whole-brain radiation, the patient underwent a resection of a symptomatic parietal lobe metastasis, which shared the same amplifications. Notably, at the time of progression in both brain metastases, there was no evidence of extracranial disease, and biopsy of an extracranial site for genetic analysis would not have been possible.
Figure 3. Anatomically and regionally distinct brain metastasis samples share actionable drivers. A–G, seven cases for which multiple regionally separated or anatomically distinct brain metastases were sequenced. The samples labeled R1, R2, etc., refer to different regions of the same pathology block. Phylogenetic trees and clinical histories are shown for each case as in Fig. 1. C and F, minor subclones shared by >1 tissue sample were detected (as described in the Methods). For these cases, the shared areas denote the tissue samples, and indicate which subclones are present in each sample. F and G, gadolinium-enhanced MRIs of the sampled brain metastases are shown.
Genomics of Brain Metastases

**Brain Metastases Are Genetically Distinct from Regional Lymph Nodes and Extracranial Metastases**

Given that brain metastases can be clinically difficult to access in some cases, we evaluated the extent to which regional lymph nodes and distal extracranial metastases were genetically similar to the brain metastases. We sequenced eight cases with at least one additional primary-tumor sample, regional lymph node, or extracranial metastasis, in addition to the paired brain metastasis (Fig. 4A–G).

The extracranial sites exhibited varying degrees of relatedness to the primary tumor and brain-metastasis samples. In four of eight cases, the number of mutations private to the brain metastasis sample was greater than the number of truncal mutations shared by all samples (Fig. 4A, C, D, and E; 402, 296, 128, 83). Notably, in case 296, broad amplification of chromosome 7 (six copies), including the **EGFR** locus, was detected in the primary-tumor sample, but not in matched samples from a regional lymph node or brain metastasis (Fig. 4C and Supplementary Fig. S18).

In 2 of 4 patients with distal extracranial metastases, the metastatic sites each harbored an approximately equal or greater number of private mutations than the number of mutations that were shared (truncal) or private to the brain-metastasis sample (Fig. 4D and E; 0128, 0083). In the third case, the clinically sampled primary tumor and lung metastasis shared a common ancestor that harbored mutations not detected in the brain-metastasis sample (Fig. 4F; 053).

In the fourth case, the brain and lung metastases shared a common ancestor not in common with the primary-tumor sample; however, the brain metastasis had more private mutations than the primary and lung metastasis combined (Fig. 4H; 0418).

In case 441, we sampled two regions of a primary lung carcinoma, one before and one after two cycles of neoadjuvant therapy.
Chemotherapy and chest radiation, in addition to a brain metastasis that was diagnosed 5 months later in the absence of any extracranial disease (Fig. 4G). The two samples from the primary tumor shared mutations that were not detected in the brain-metastasis sample, and the brain metastasis harbored mutations of uncertain significance in ALK (P254H), FBXW7 (R357T), and FAT1 (R2041fs) that were not detected in either primary-tumor samples (Fig. 4G).

**DISCUSSION**

Brain metastases represent an unmet need in current oncologic care. Approximately 8% to 10% of patients with cancer will develop brain metastases, and more than half of these patients will die within a few months following diagnosis of intracranial metastasis (1). Genomically guided clinical trials have been successful at matching patients to novel targeted agents in patients with advanced cancer; however, patients with active brain metastases are routinely excluded from these trials in part due to the poor correlation between systemic response and brain response (1). Patients will often develop progressive brain metastases in the setting of extracranial disease that is adequately controlled with existing chemotherapies or targeted therapies. Historically, this clinical divergence has been ascribed to inadequate systemic therapeutic penetration of the blood–brain barrier. The observations presented here suggest that additional potentially oncogenic alterations may be present in brain metastases, and might contribute to this divergence of therapeutic response in some of these cases.

We note that these mutations may represent precursors in the evolutionary process leading to the metastasis; for example, they may have driven the proliferation or survival of a premetastatic subclone within the primary tumor (that was not sampled clinically). Alternately, it is possible that some of these alterations were necessary for the establishment of the initial metastatic outgrowth in the brain, but not for its continued growth or maintenance. In addition, we note that it is possible that some of the dependencies associated with these alterations may be histology specific or dependent on the presence or absence of additional mutations. As our study involved a retrospective collection of samples, further prospective clinical studies with agents that cross the blood–brain barrier will be required to demonstrate that these mutations are viable therapeutic targets for patients with brain metastases.

We found that 46 of 86 (53%) patients harbored a potentially clinically actionable alteration in the brain metastasis that was not detected in the clinically sampled primary tumor (Fig. 2). These alterations may have critical clinical implications because (i) patients often develop brain metastases even when presumably truncal mutations identified in the primary tumor are successfully targeted with active systemic agents [e.g., BRAF inhibitors (42), ALK inhibitors (43), or HER2 inhibitors (44)]; (ii) additional evolution in the brain metastasis lineage might contribute to treatment resistance; (iii) actionable mutations present in the brain metastasis cannot be reliably identified on the basis of only a single biopsy of the primary tumor (Fig. 2); and (iv) the primary and metastatic cancer samples may be clonally unrelated, as was the case in four of the 86 cases in our study. Because more than 50% of patients with brain metastases will die of intracranial progression, targetable alterations present in cancer subclones specific to the brain metastasis represent an important opportunity for novel targeted therapeutic strategies to affect overall survival.

Tissue from craniotomies provides an immediate opportunity for more informed decision-making based on genomic analysis. Many patients will have a brain metastasis resected as part of clinical care. Current clinical indications for craniotomies in brain metastases include: need for histologic diagnosis; resection of single (25%–50% of brain metastases; refs. 45–47) or oligometastatic disease in the setting of controlled extracranial disease; or resection of a symptomatic or dominant lesion in the setting of multiple brain metastases. Here, we show that although genetically divergent from samples of their primary tumor (Figs. 1 and 2), intracranial metastases were remarkably homogeneous with respect to driver and/or potentially targetable alterations (Fig. 3), a finding with implications for the metastatic tropism of evolutionary branches that arise early during neoplastic development. Practically, this homogeneity implies that, when clinically available, characterization of even a single brain metastasis lesion may be more informative than that of a single primary tumor biopsy for selection of a targeted therapeutic agent. Notably, regional lymph node and distal extracranial metastases were not reliable surrogates for the oncogenic alterations found in brain metastases (Fig. 4).

We note that more comprehensive characterization of the primary tumor might reveal subclones that more closely resemble intracranial disease. In current clinical practice, however, decisions are often made after bulk molecular analysis of only a single biopsy from the primary tumor; without a sample of brain metastasis tissue it is impossible to determine to what extent genetic alterations in the primary biopsy represent the divergent evolutionary branch of brain metastases. In future studies, analysis of circulating tumor cells or cell-free DNA (from either blood or cerebrospinal fluid) should be assessed in the context of existing brain-metastasis tissue and autopsy studies in order to establish to what extent they might be informative regarding actionable genomic alterations in brain metastases.

**METHODS**

The study was reviewed and approved by the human subjects Institutional Review Boards of the Dana-Farber Cancer Institute (Boston, MA), Brigham and Women’s Hospital (Boston, MA), Broad Institute of Harvard and MIT (Boston, MA), Massachusetts General Hospital (Boston, MA), Seoul National University College of Medicine (Seoul, South Korea), and Vall d’Hebron University Hospital (Barcelona, Spain). The study was conducted in accordance with the Declaration of Helsinki. Written informed consent was obtained from all participants. We identified 104 matched brain metastases, primary tumors, and normal tissue that were collected as part of standard clinical care between 1998 and 2012. In 15 of these cases, we collected additional samples including multiple brain metastasis lesions (7 cases) and extracranial lesions (8 cases with regional lymph node metastases, extracranial metastases, or additional primary-tumor tissue). All patients provided written informed consent for genetic analysis. Board-certified neuropathologists (S. Santagata, A. Stemmer-Rachamimov, and D.N. Louis) confirmed the histologic diagnoses and selected representative fresh-frozen or formalin-fixed
paraffin-embedded samples that had an estimated purity of ≥ 40%. We performed whole-exome sequencing of extracted tissue using methods as described on Illumina HiSeq or Genome Analyzer IIx platforms (48, 49). Samples were sequenced to median average depth of 108.3X (Supplementary Fig. S19). Of the 104 cases, we focused on the 86 (Supplementary Table S1) that exhibited sufficiently high purity in both the primary and brain-metastasis samples (16) and for which the DNA libraries were of sufficient quality (Supplementary Fig. S19 and Supplementary File S1). Somatic copy-number alterations were inferred from sequencing read depth (Supplementary Fig. S8, S9, S12-S18, S20, and Supplementary File S2). In addition, we performed deep targeted sequencing (median depth 45X) on a subset of primary-tumor samples using the Illumina HiSeq platform (50) to confirm the presence or absence of mutations (Supplementary Table S2). Immunohistochemistry for HER2/NEU overexpression was used to validate amplification of ERBB2 in the brain metastasis and primary tumor in case 331.

Additional details regarding materials and methods are provided in the Supplementary Methods.

Accession codes: All data have been deposited in the database of Genotypes and Phenotypes (dbGaP); accession number phs000730.v1.p1.

Analysis codes: Source-code implementing methods used in this article can be accessed at http://bcb.dfci.harvard.edu/~scarter/clonal_evolutionsuite.

**Branched-Sibling Model**

In order to address the genetic heterogeneity of cancer-tissue samples, we analyzed mutation CCF data to determine whether the tissue samples were sufficiently diverged from one another such that no detectable overlap of minor subclones (CCF < 1) occurred, a scenario we term the branched-sibling model (Supplementary Figs. S7B, S17A-S17C). In this model, the related cancer-tissue samples descend from a common ancestral clone, but each has continued to evolve independently with no overlap of subclones in the sampled tissues. In this scenario, it is valid to construct standard phylogenetic trees relating each tissue sample, with minor subclones (CCF < 1) private to each tissue sample represented as subbranches grafted on to each sample tip. The branched-sibling scenario implies that such trees accurately represent the evolutionary relationship of all subclonal populations detected with CCF = 1 in the sampled cancer tissues. A corollary of the branched-sibling model is that all mutations shared in two or more samples must have CCF = 1 wherever they are present. Thus, the appearance of mutations shared in two or more samples with CCF < 1 in any of them either represents technical artifact or constitutes evidence that the branched-sibling approximation is not an accurate description of those samples. Because some degree of technical artifact is occasionally expected due to either sequencing errors or incorrect estimation of CCF values, we applied further logical constraints on the phylogenetic relationships between subclones in order to distinguish true violations of the branched-sibling scenario (described below).

To analyze the evolutionary relationship between paired primary-tumor and brain-metastasis samples, we first examined whether we could find any cell population in any primary-tumor sample that was an ancestor of the metastasis. Such a metastasis-founding subclone would harbor mutations in a subset of the cancer cells of the primary-tumor sample (CCFprimary < 1) that were present in all cancer cells (CCFmet = 1) of the metastasis sample (violating the branched-sibling model; Supplementary Fig. S7C). For each patient, we analyzed the two-dimensional CCF distributions of point mutations for all unique tissue-sample pairs (Supplementary Figs. S1 and S3 and Supplementary File S3) using a previously described 2-D Bayesian clustering algorithm (ref. 19; Supplementary Methods). In most patients, we observed some mutations with CCFprimary = 1 that were not detected in the paired metastasis. We reasoned that, because subclones defined by CCFprimary < 1 and CCFmet = 1 must be the evolutionary siblings of subclones defined by CCFprimary < 1 and CCFmet = 0, a metastasis-founding subclone could not have been present at a detectable fraction in these primary-tumor samples, as this subclone would have displaced the mutations exclusive to the primary, so that none would have CCFprimary = 1 (Supplementary Fig. S7B). Thus, the observation of mutation clusters with CCFprimary < 1 and CCFmet = 1 in the absence of this displacement was not considered to be convincing evidence for a branched-sibling violation (Supplementary File S3). We recently applied similar analysis to data from a mouse model of lung cancer (20), where a valid metastasis-founding subclone was detected (Fig. 5 therein); however, we note that approximately 50% of the total tumor mass was harvested for sequencing in that case.

Following similar reasoning, we examined CCF values in all pairs of related cancer-tissue samples. Most sample-pairs exhibited robust mutation clusters with CCF = 1 in one sample that were undetected in the other (Supplementary File S3), implying that they were sufficiently diverged from one another such that no partial-sharing of subclones occurred between them. We note that evidence supporting partial sharing of subclones between multiple sequenced regions of individual brain metastases was observed for some cases, necessitating special treatment (described below).

**Phylogenetic Inference on Related Cancer-Tissue Samples**

We created phylogenetic trees using a four-phase process in order to (i) be robust to both false-positive and false-negative mutation calls; (ii) assign mutations to the correct branches of the tree; (iii) distinguish tissue-restricted minor subclones, present in only a subset of the cancer cells in a given sample (CCF < 1); and (iv) identify cases where minor subclones were shared by two or more related tissue samples (violating the branched-sibling model) and correct the phylogenetic trees accordingly.

In the first phase, we sought to find the best phylogenetic tree explaining the observed point-mutation data. Somatic point-mutations were assumed to have arisen uniquely during the clonal evolution of the cancer, with negligible back-mutation rates, for example, due to chromosomal deletion of mutated alleles, which did not appear to help explain the data (not shown). We constructed a binary matrix of present/absent values for all point mutations detected in any of the samples analyzed from a given patient. For each sample, absent sites for which paired-detection power was <0.7 were removed from consideration, as were sites for which <3 reads supporting the mutation were observed. We then searched for the maximum-parsimony phylogeny using the parsimony-ratchet method (51) on this matrix.

In the second phase, we sought to assign mutations to branches of the phylogeny inferred in phase I, taking into account uncertainty in the provisional mutation forced calls. We applied the Bayesian clustering procedure described in the Supplementary Methods to each sample individually, retaining all mutations provisionally called with >0 supporting reads in that sample. A single pseudo-count observation was added having CCF = 1. We then identified all provisional mutation calls (>0 supporting reads) made in at least two samples of the case that were assigned to a CCF cluster with posterior mode < 1.0 (Supplementary Fig. SSA). These mutation calls, which appeared to violate the branched-sibling model (described above), were then rejected if the number of supporting reads was <3 (Supplementary Fig. SSB). This modified matrix of mutation calls was then used to assign each mutation to a branch of the phylogenetic tree by assuming that the mutation occurred uniquely during clonal evolution and was not subject to back mutation. For each sample, the number of mutations in each category is shown in Supplementary Fig. SSC. Assignment of gene-level SCNAs to branches was performed in a similar manner in the primary tumor (Supplementary Fig. SSD).

In the third phase, we sought to obtain a more complete description of the genetic divergence between the various tissue samples of...
each case. We refined the tips of each phylogenetic tree by distin-
guishing between private mutations that occurred in all cancer cells of
each sample (CCF = 1) versus those that occurred in a restricted
subset of sampled cancer cells (CCF < 1). To make this distinc-
tion, for each sample, we applied the Bayesian clustering technique
(described in Supplementary Methods) to the private mutations
called only in that sample. We added N pseudo-count observa-
tions of CCF = 1, where N was the number of mutations called in
>1 samples of the case that were also called in the sample being
considered. This process partitioned the private mutations into
a small number of putative subclones having distinct CCF values
(Supplementary Fig. S4). We then modified the phylogenetic trees
by replacing each (non-germline) tip with a subtree representing the
maximally branching microphylogeny consistent with the observed
set of CCF-cluster values (i.e., respecting the rule that the sum of
sibling subclones cannot exceed that of their most recent common
ancestor; Figs. 1, 3, and 4, and Supplementary Fig. S6).

In the fourth phase, we examined whether evidence that the
branched-sibling model was not an adequate approximation of the
sampled cancer tissues could be discerned. We manually reviewed
detailed plots (Supplementary File S3) showing the estimated CCF
value of each mutation in each tissue sample, as well as the 2-D clus-
tering results of mutation CCF values in all unique pairs of related
tissues samples (Supplementary Fig. S3) for evidence of minor sub-
clones (CCF < 1) shared by two or more samples, as described above.
In two cases in which evidence contradicting the branched-sibling
model was observed, phylogenetic trees were manually adjusted (as
described below) to accurately reflect the evolutionary relationship
between the different clonal lineages as shown in Fig. 3C and F. This
was done in a manner analogous to that described in a recent report
(20); here, we extended similar logic to the scenario where the same
subclone was present in multiple sequenced tissue samples. Detailed
analysis of mutation CCFs for each patient, including the automati-
cally generated phylogenetic trees (prior to manual adjustment), are
available in Supplementary File S3.

For patient 138 (Fig. 3F), samples BM1 region 1 and BM1 region 2
shared a minor subclone (subclone1) defined by 15 mutations,
present at CCF = 0.6 in BM1 region 1 and CCF = 0.55 in BM1 region
2. Because the mutations private to these samples had CCF values
consistent with being the siblings of subclone1 (CCF = 0.1 in BM1
region 1 and CCF = 0.3 in BM1 region 2), we redeployed the tree this way.

For patient 314 (Fig. 3C), samples BM region 2 and BM region 4
shared a minor subclone (subclone 2), defined by eight mutations,
present at CCF = 0.45 in BM region 2 and CCF = 0.35 in BM region
4. Samples BM region 1 and BM region 3 shared a minor subclone
(subclone 1), defined by seven mutations, present at CCF = 0.55 in
BM region 3 and CCF = 0.4 in BM region 1. In addition, BM region 1
and BM region 3 appeared to contain a small number of cells (CCF < 0.05)
from subclone 2. In addition, extreme heterogeneity of primary-tumor
sample may have resulted in inaccurate CCF values for some muta-
tions, leading to the appearance of a cluster having CCF < 1 in the
primary and CCF = 1 in all metastatic samples.

Patients 176, 302, and 137 showed some evidence consistent with
shared subclones, but due to the small number of mutations involved
and the uncertainty in their CCF values, judgments about the validity
of these branched-sibling violations could not be made with confi-
dence. The trees were therefore left unaltered.

In addition, patients 331, 104, 52, 263, and 91 harbored shared
mutations with CCF < 1. However, they were not logically consistent
with true violations of the branched-sibling model (e.g., they failed
to displace private mutations, which were present at CCF = 1 in
most samples from these cases). This, coupled with the substantial
hetereogeneity of the copy profiles in some of these samples, led us
to consider the appearance of some mutations appearing to violate
the branched-sibling model due to incorrect estimation of CCF values.

Prioritization of Clinically Informative Mutations
Using TARGET

To systematically evaluate somatic alterations of potential clinical
interest, we used the TARGET database (31) of genes for which somatic
alterations have therapeutic or prognostic implications in at least one
tumor type (Supplementary Table S3). Because the therapeutic or pro-
gnostic evidence in TARGET is often based on one or a few tumor types,
we currently do not have evidence that these events will be predictive
of clinical responses to the indicated targeted therapeutic agent in all of
the tumor types studied here. Ongoing clinical trials to test such hypotheses
(“basket trials”) accept any patient with a particular alteration regardless
of their primary histology. However, there is evidence that in some cases,
such as for BRAF V600E mutations in colorectal cancer, the responses
to therapies targeting the same genomic events are histology dependent.

Alterations in TARGET genes were prioritized according to predefined
criteria (31). For example, some genes were required to have biallelic
inactivation, whereas others required amplification or specific point
mutations. In order to nominate a mutation as “potentially clini-
cally informative,” we first distinguished between heterozygous and
homozygous events (in which no reference alleles remained in the can-
cer cells), by analyzing read-counts at mutated loci using ABSOLUTE
(16) to account for genomic copy numbers and sample purity.

We accepted as fulfilling the “biallelic inactivation” TARGET crite-
ria genes harboring homozygous loss-of-function (LOF) mutations,
homozygous deletion, or two heterozygous LOF mutations. LOF
mutations were defined as: nonsense, frame-shift indel, in-frame
indel, or splice-site mutations. To satisfy the “mutation” TARGET
criteria, we required the presence of at least one identical amino acid sub-
stitution in the Catalogue of Somatic Mutations in Cancer (COSMIC)
database (67–69, ref. 52). To satisfy the “amplification” TARGET criteria,
we required a gene-level somatic copy-number alteration call of either
“amplification” or “high-level amplification” (as described above).

Disclosure of Potential Conflicts of Interest

E.M. Van Allen is a consultant/advisory board member for Syspe
and Roche Ventana. B.E. Johnson has ownership interest (including
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Genomics of Brain Metastases

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Other (providing fruitful discussions about the interpretation of results): M.P. Hoang

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RESEARCH BRIEF

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