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Profiles of brain metastases: Prioritization of therapeutic targets

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We sought to compare the tumor profiles of brain metastases from common cancers with those of primary tumors and extracranial metastases in order to identify potential targets and prioritize rational treatment strategies. Tumor samples were collected from both the primary and metastatic sites of nonsmall cell lung cancer, breast cancer and melanoma from patients in locations worldwide, and these were submitted to Caris Life Sciences for tumor multiplatform analysis, including gene sequencing (Sanger and next-generation sequencing with a targeted 47-gene panel), protein expression (assayed by immunohistochemistry) and gene amplification (assayed by in situ hybridization). The data analysis considered differential protein expression, gene amplification and mutations among brain metastases, extracranial metastases and primary tumors. The analyzed population included: 16,999 unmatched primary tumor and/or metastasis samples; 8,178 nonsmall cell lung cancers (5,098 primaries; 2,787 systemic metastases; 293 brain metastases), 7,064 breast cancers (3,496 primaries; 3,469 systemic metastases; 99 brain metastases) and 1,757 melanomas (660 primaries; 996 systemic metastases; 101 brain metastases). TOP2A expression was increased in brain metastases from all 3 cancers, and brain metastases overexpressed multiple proteins clustering around functions critical to DNA synthesis and repair and implicated in chemotherapy resistance, including RRM1, TS, ERCC1 and TOP01. cMET was overexpressed in melanoma brain metastases relative to primary skin specimens. Brain metastasis patients may particularly benefit from therapeutic targeting of enzymes associated with DNA synthesis, replication and/or repair.

Key words: brain metastases, molecular profiling, multiplatform analysis, DNA repair enzymes, TOP2A

Additional Supporting Information may be found in the online version of this article.

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Brain metastases are difficult to treat and generally lethal. Here, the authors sought clues to possible treatment targets by comparing the molecular characteristics of lung, breast, and skin cancers with those of brain metastases. They analyzed DNA sequence, protein expression, and gene amplification on over 17,000 samples, the largest metastases cohort ever studied. They found that the enzyme TOP2A, involved in DNA transcription, was enriched in the metastatic cells relative to the primary tumor cells. Other enzymes, involved in DNA synthesis, repair, and replication were also overexpressed in metastases, and could potentially be useful therapeutic targets.

Introduction
Metastasis is the most common source of tumors in the brain, with lung cancer, breast cancer and melanoma being the most common solid tumors spreading to the central nervous system (CNS).1 Overall, it is estimated that 9–17% of cancer patients will develop brain metastases during the course of their disease and that approximately half will die within 3–27 months from initial diagnosis of CNS involvement.1–3 The reported median survival times for NSCLC, breast cancer and melanoma after the diagnosis of brain metastasis are 7.8 months, 15 months and 7–7.9 months, respectively.4–6 The poor survival associated with brain metastases is largely attributed to our incomplete understanding of the molecular mechanisms governing organotropism and the brain itself being a known sanctuary site. Additionally, most systemic therapeutics are not capable of crossing the blood–brain barrier (BBB). The need for additional treatment options is urgent; more than 100,000 patients per year develop brain metastases in the United States, and the current treatment cost exceeds 3 billion dollars.7 Current treatment options include surgical resection and various forms of irradiation, but these have only a modest impact on overall outcome and carry variable risks of neurological morbidity. There are no effective systemic therapies specifically approved for brain metastases. Unfortunately, once CNS involvement is present, effective treatment options are limited and the prognosis is dismal. These issues are pressing, as the incidence of brain metastasis may be increasing due to longer patient survival owing to newer systemic therapies that control extracranial disease, along with more frequent magnetic resonance imaging surveillance.3,5

There has been a surge in clinical trials guided by genomics in order to better match patients with effective, targeted cancer treatments.8 The development of CNS metastases often results in exclusion of patients from clinical trials, particularly phase I trials of many novel therapeutics, mainly due to the known poor prognosis and limited efficacy of systemic therapy for these patients historically. Detailed molecular analyses of metastases across pathologies may help identify new therapeutic targets that can benefit these patients. Thus, we performed a multiplatform analysis of a large cohort of brain and systemic metastasis samples collected from the cancers most commonly predisposed to produce brain metastases in order to identify potentially actionable targets.

Materials and Methods
Study population
From 2009 to 2015, tumors collected from both the primary and metastatic sites of nonsmall cell lung cancer (NSCLC), breast cancer and melanoma from patients in locations worldwide were submitted to Caris Life Sciences for tumor multiplatform analysis. A total of 8,178 NSCLC (5,098 primaries; 2,787 systemic metastases; 293 brain metastases), 7,064 breast cancer (3,496 primaries; 3,469 systemic metastases; 99 brain metastases) and 1,757 melanoma (660 primaries; 996 systemic metastases; 101 brain metastases) samples (primary and metastases not matched to the same patient) were retrospectively analyzed. Our study is exempt per policy 45 CFR 46.101 (b); the data analyzed is from an existing commercial repository and subject information is de-identified.

Immunohistochemistry
All analysis by immunohistochemistry (IHC) was performed on full slides of formalin-fixed paraffin-embedded (FFPE) tumor specimens. Following the requirements of the Clinical Laboratory Improvement Amendments (CLIA)/Compliance Assistance Office (CAO) and International Organization for Standardization (ISO), staining conditions were performed per the manufacturer’s instructions using automated staining techniques and were optimized and validated. The results were evaluated and confirmed by independent board-certified pathologists. Immunohistochemical scoring was based on staining percentage (0–100%) and intensity (0 = no staining; 1 += weak staining; 2+ = moderate staining; 3+ = strong staining). Representative staining photos are shown in Supporting Information Figure S1. Results were categorized as positive or negative by defined thresholds specific to each marker based on published clinical literature that associates biomarker status with patient responses to therapeutic agents. Supporting Information Table S1 shows the thresholds for positive and negative categorization. Tumor cells were evaluated for proteins of interest. Analyzed proteins and antibodies used are listed in Supporting Information Table S2. PD-L1 staining results are presented and categorized in Supporting Information Table S5.

In situ hybridization (ISH)
Gene amplification was assessed using fluorescence in situ hybridization (FISH) for EGFR (EGFR/CEP7 probe) and TOP2A (TOP2A/CEP17 probe). Both FISH and chromogenic
in situ hybridization (CISH) were used to detect gene amplification of cMET (cMET/CEP7 probe and Ventana kit) and HER2 (HER-2/CEP17 probe and INFORM HER-2 Dual ISH DNA Probe Cocktail). EGFR amplification was defined by the presence of an EGFR/CEP7 ratio of ≥2, or ≥15 EGFR copies per cell in ≥10% of analyzed cells. A ratio of TOP2a:CEP17 ≥ 2.0 and an HER-2/CEP17 ratio ≥ 2 was considered amplified; cMET was considered amplified if ≥5 copies per tumor cell were detected on average.

Mutational analysis
Using the Illumina MiSeq platform, sequencing was performed on genomomic tumor DNA isolated from FFPE tumor tissue (germline DNA was not sequenced). Reference genome used was hg19 from the UCSC Genome Browser database (http://hgdownload.cse.ucsc.edu/goldenPath/hg19/bigZips/). Specific regions of 47 genes (panel of pan-cancer genes of interest related to cancer genomics based on current literature) were amplified using the customized Illumina TruSeq Amplicon Cancer Hotspot panel.11 All variants reported were detected with >99% confidence based on the mutation frequency present. Mutations including PTEN, TP53 and BRAF and were evaluated by Next-generation sequencing (NGS; Illumina MiSeq platform) (Supporting Information Table S3). The average depth of coverage of the hotspot panel was >1,500x and the tumor content requirement is ≥20%. This test has a sensitivity to detect as low as a 10% population of cells containing a mutation in a sequenced amplicon. All variants reported were detected with >99% confidence based on the frequency of the mutation present and the amplicon coverage. Note that as this is a commercial repository, mutational testing on individual samples was performed at the discretion of the ordering physician. Hence, not all samples were tested for all mutations. Additionally, a cohort of patients was profiled prior to the availability of NGS.

Statistical analysis
Comparisons of biomarker profiles, including protein expression (IHC), gene amplification (CISH/FISH) and mutation (Sanger sequencing and NGS) between tumor sites were performed using Fisher’s exact test. To adjust for the multiple testing and limit the risk of false positive results, p values resulting from the Fisher’s exact test were corrected using the Benjamini-Hochberg procedure.12 All reported p values are two sided and corrected for multiple comparison. P values of less than 0.05 were declared as statistically significant. All analyses were performed with statistical software R v3.3.1.

Results
Study population
Patient characteristics, number of tumor specimens and distribution of systemic metastases (extracranial) are shown in Table 1 and Supporting Information Table S4. A total of 16,999 specimens were included for analysis; 9,252 primary lesions, 493 brain metastases and 7,252 systemic (extra-cranial metastases). The majority of extracranial specimens were obtained from the lymph nodes. Sex was equally dichotomized among the NSCLC patients, breast cancer cases were mostly restricted to females and males were overrepresented in melanoma. Overall, the breast cancer patient population was younger than the NSCLC and melanoma cohorts.

TOP2A expression is a high frequency target in brain metastases relative to primary tumors
Using a CLIA-compliant cancer protein panel, differential expression was profiled in NSCLC, breast cancer and melanoma specimens (Fig. 1, Supporting Information Table S5). Across pathologies, TOP2A, which controls and alters the topologic states of DNA during transcription, was the protein expressed more frequently in the brain metastases relative to the primary tumors most consistently (NSCLC: 74.5% versus 55%, p < 0.0001; breast cancer: 78.8% versus 50.1%, p = 0.0001; melanoma: 76.4% versus 45.8%, p = 0.0007) (Fig. 2). Levels of two other enzymes involved in DNA synthesis and chemoresistance, RRM1 and TS, were also increased in NSCLC brain metastases relative to the primary (31.8% versus 20.9%, p = 0.0049; and 34.9% versus 21.5%, p = 0.0006, respectively), but this was only a trend for increased expression detected in breast cancer and melanoma brain metastases. Other DNA topoisomerases and enzymes essential for DNA replication/repair such as TOP1 (77.9% versus 63.4%, p = 0.0357) and ERCC1 (61.8% versus 42.1%, p = 0.0199) were also more frequently expressed in breast cancer brain metastases. Her2/Neu expression was more common in breast cancer brain metastases (23.3%) compared with primary specimens (11.6%) (p = 0.0227), whereas the frequency of expression of all hormonal receptors (AR: 20.5% versus 30.9%, p = 0.0001; and PR: 25.3% versus 43.6%, p = 0.008) was significantly reduced in brain metastases relative to the primary tumor. Melanoma brain metastases exhibited increased cMET expression frequency relative to the primary (36.4% versus 7.9%, p = 0.0003) but decreased TUBB3 expression (38.2% versus 76.2%, p = 0.0007).

Her2/Neu protein expression is more frequent in breast cancer brain metastases than in systemic metastases
To ascertain whether the differences in protein expression were a function of the process of metastasis in general or were more specific to brain metastasis, we next compared differential protein expression in brain and systemic metastases across tumor pathologies (Fig. 1, Supporting Information Table S5). Her2/Neu expression was significantly higher in breast brain metastases (23.3%) than in systemic metastases (9.9%) (p = 0.0044). Moreover, all hormone receptors were expressed less frequently in breast cancer brain metastases (AR, p < 0.0001; ER, p = 0.0001 and PR, p = 0.019)—similar to the comparison with primary sites. Systemic breast metastases showed an increased frequency of PTEN (52.7% versus 34%, p = 0.0049) and MGMT (68% versus 47.3%, p = 0.0011) relative to brain metastases (Fig. 2).

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Breast cancer brain metastasis demonstrated enrichment of Her2 and EGFR gene amplifications relative to systemic metastases and primary lesions

Gene amplification results for NSCLC, breast cancer and melanoma are presented in Table 2. A total of 2,888 breast cancer specimens were evaluated for Her2 amplification using FISH (2,808 breast primaries), with 28.8% (n = 80) of brain metastases displaying Her2 amplification relative to 15.3% (n = 430) of the primary specimens (p = 0.0227). Her2 amplification was also significantly higher in breast cancer brain metastases than in systemic metastases (13.1%) (p = 0.004), consistent with HER2-expressing cells having a greater propensity to spread to the brain.13,14 Furthermore, EGFR amplification was more common in breast cancer brain metastases (31.1%) than in primaries (14.1%; p = 0.0341). Similarly, EGFR amplification was higher in brain metastases than in systemic lesions (14.2%; p = 0.0333). EGFR was also amplified in melanoma brain metastases (50%) and NSCLC brain metastases (36.3%) relative to the primary cancers (6.1% and 28%, respectively), but these differences were not statistically significant.

Minimal differences in mutational frequency between metastases and primary lesions

DNA sequencing data were analyzed for a panel of 47 well-annotated pan-cancer genes (listed in Supporting Information Table S6). In most cases, the most common mutations were found in both the primary cancers and brain metastases with...
similar frequencies. However, there were some notable exceptions in breast cancer. For example, ABL1 mutations were more frequent in breast cancer brain metastases (11.1%; 2/18) than in primary breast cancers (0.4%; 3/822) ($p = 0.0319$) and PIK3CA mutations were more frequent in breast systemic metastases (30.3%; 415/1,370) than in brain metastases (10.3%; 4/39) ($p = 0.0426$) (Fig. 3; Supporting Information Table S7).

Discussion

Multiplatform analysis was performed on approximately 17,000 primary and metastatic cancers, which is the largest cohort described in the literature, in order to reveal potential therapeutic targets. We observed a significant association between TOP2A protein expression and brain metastases. TOP2A is a critical enzyme in DNA transcription and regulation that is highly expressed in proliferating cells, as such we cannot confirm this finding is not influenced by higher proliferative activity\(^{15}\) TOP2A overexpression is associated with an aggressive cancer phenotype and decreased patient survival,\(^{16-18}\) which makes it an attractive therapeutic target. Unfortunately, most currently available TOP2A inhibitors have limited BBB penetration, making them ineffective for treating brain metastases.\(^{19}\) We did not observe TOP2A amplification in brain metastases, however it is established that TOP2A protein expression is not secondary to gene amplification, which likely explains this discordance.\(^{20}\)

The proteins RRM1, ERCC1 and TS (involved in DNA replication, repair and chemotherapy resistance) were more frequently expressed in metastases than in primary lesions across all three tumor pathologies (NSCLC, breast cancer and melanoma). The DNA repair enzyme ERCC1, which plays a role in therapeutic resistance to platinum-based chemotherapy,\(^{21,22}\) has not been previously documented to be associated with
breast cancer brain metastases. Similarly, the DNA synthesis protein RRM1, which is linked to chemosensitivity to fluoropyrimidine and gemcitabine-based chemotherapy,\textsuperscript{23} and TS,\textsuperscript{24} were shown to have increased expression in NSCLC brain metastases. This expression may be a result of the development of therapeutic resistance mechanisms to prior therapy or an intrinsic feature of rare tumor clones in the primary that have an increased propensity for aggressive behavior, including metastasis. A recent single institution study reported the genomic analysis of 500 metastatic cancer patients and also reported a notable incidence of defects in DNA repair in concordance with our findings.\textsuperscript{25}

Consistent with the literature, we identified less frequent expression of hormone receptor expression in breast cancer brain metastases,\textsuperscript{26–28}—although in a much larger patient cohort and with the novel extension of this to androgen receptor (AR) status. Hormone receptor loss is a known marker of aggressive disease, negative prognosis and limited treatment options. It has been suggested that the loss of hormone receptor expression is due to dedifferentiation of the metastatic tumor\textsuperscript{29} or the selection of cytogenetically unique clones that have an enhanced propensity to metastasize to distant sites.\textsuperscript{30,31} Alternatively, the loss of hormone receptor expression in breast cancer patients may represent selection bias as a result of treatment.\textsuperscript{32}

Brain metastasis is a late event in hormone receptor-positive breast cancer. As a breast cancer loses its hormone sensitivity, it may behave more like triple-negative breast cancer, known to have a higher rate of brain metastasis and a worse prognosis.\textsuperscript{33} Because our study did not employ paired primary tumor/metastasis samples, we cannot exclude the possibility that our brain metastasis cohort is enriched for triple-negative cases.

Specific to melanoma brain metastases was the increased expression of cMET, which is a receptor tyrosine kinase that binds HGF, thereby leading to the upregulation of oncogenic signaling cascades. The association of cMET with tumor progression and brain metastasis has been previously documented in animal models using \textit{cMET} knockdown demonstrating decreased occurrence of brain metastases by 70%.\textsuperscript{34–36} Therapies targeting \textit{cMET} via the use of monoclonal antibodies are in various stages of clinical trial testing in multiple cancer histologies,\textsuperscript{37} but the efficacy of such drugs in the central nervous system is unclear (NCT02414139, NCT02468661). Another therapeutic consideration in this setting would be cabozantinib, which is being evaluated in breast cancer patients with brain metastasis (NCT02260531).

PD-1 expression was very frequent in both brain metastases and the primaries of NSCLC and melanoma cancers. The interaction between the immune system, tumor and the tumor microenvironment has been the focus of intense investigation—specifically the role of the PD-1/PDL-1 signaling axis.\textsuperscript{38} The efficacy of PD-1 inhibition (i.e., nivolumab and pembrolizumab) as a monotherapy or in combination therapy has shown favorable survival in clinical trials for patients with advanced melanoma and NSCLC.\textsuperscript{39–43} However, the role of immune checkpoint inhibitors in the treatment of CNS cancers, including metastasis, is currently being defined,\textsuperscript{44,45} and our current results provide further support for these agents in this context.

Although most genetic alterations were present at similar frequencies in the brain metastases and the primary tumors, there were some notable exceptions that were higher in the brain metastasis. \textit{ABL1} mutations and \textit{HER2} and \textit{EGFR} amplification were common in breast cancer brain metastases, consistent with a prior study of whole-exome sequencing of 86 matched primary tumor/brain metastasis samples from multiple cancers.\textsuperscript{46} In previous study, clinically actionable alterations not detected in matched primary tumors were found in more than 50% of brain metastases. Our study\textsuperscript{46} also included matched samples and tumor pathologies that were not in our data set (e.g., renal cell carcinoma), some of which were highlighted for their alterations specific to brain metastases. It is also possible that in our

| Gene        | NSCLC Brain | NSCLC Primary | NSCLC Systemic | BREAST CANCER Brain | BREAST CANCER Primary | BREAST CANCER Systemic | MELANOMA Brain | MELANOMA Primary | MELANOMA Systemic |
|-------------|-------------|---------------|----------------|---------------------|-----------------------|------------------------|----------------|----------------|----------------|
| \textit{EGFR} | 36.3%       | 28.0%         | 34.5%          | 31.1%\textsuperscript{2} | 14.4%                 | 14.2%\textsuperscript{4} | 50%\textsuperscript{4} | 6.1%           | 28.9%          |
| \textit{TOP2A} | 0%          | 5%            | 1.6%           | 16.7%               | 8.5%                  | 6.3%\textsuperscript{3}  | 0%             | 0%             | 0%             |
| \textit{Her2}  | 5.8%        | 3.6%          | 3%             | 28.8%\textsuperscript{2} | 15.3%                 | 13.1%\textsuperscript{4} | 0%             | 0%             | 0%             |
| \textit{cMET}  | 6.4%        | 4%            | 5.2%           | 5.6%                | 1.1%                  | 0.7%                   | 8.6%\textsuperscript{3} | 0.6%           | 3.5%           |

NSCLC, nonsmall cell lung cancer.
\textsuperscript{1}Bivariate analysis \textit{p} < 0.05 (brain metastases relative to primary).
\textsuperscript{2}Corrected for multiple testing \textit{p} < 0.05 (brain metastases relative to primary).
\textsuperscript{3}Bivariate analysis \textit{p} < 0.05 (systemic metastases relative to brain metastases).
\textsuperscript{4}Corrected for multiple testing \textit{p} < 0.05 (systemic metastases relative to brain metastases).
analysis, we were not powered to detect mutations present at a low frequency (subclonal events). Yet, our sample size was very large, which allowed for rigorous statistical analysis. Comparing the findings of the current study to smaller cohorts of published samples is challenging as the majority of studies focus on muta-
tional changes/alterations in oncogenic signaling pathways while the major findings in our study focus on differential protein expression.\(^{25,48}\) For example, a study comparing marker profiles of the 43 tumors that had evaluable material from matched sets of primary breast cancer and brain metastases showed no statistically significant difference between the primary and metastatic breast carcinomas for any markers.\(^{49}\) However, these authors did not assess for several of the markers evaluated in our study, which were found to be significantly higher in breast cancer brain metastases (i.e., TOP2A, TOPO1, ERCC1). An additional study by Thompson et al. compared 41 matched breast and brain metastases. These authors reported significant differences in p27kip1 and cyclin D1 expression between brain and primary lesions; however, they also did not assess the expression of the markers listed above.\(^{50}\)

Although our study is strengthened by its large sample size and wide array of markers analyzed, it has limitations. To collect such a large volume of data, a commercial database was utilized and clinical annotation is not available, including prior treatment regimens. Hence, we cannot account for the impact of treatment-related changes on the patterns of expression in the brain metastasis samples. Furthermore, this analysis does not clarify whether the differentially expressed proteins are the etiology for the development of brain metastasis or are a reflection of the unique brain microenvironment, which would be informative for preventive strategies. Additionally, we cannot exclude the possibility that tumors from different sites may represent variations in molecular subtype or different stages of the disease. Ideally matched samples would better resolve these questions; however, matched samples collected from multiple centers worldwide from a cohort size of this magnitude is not feasible and is a major challenge even at a single institution.\(^{25}\) Yet, this is the largest data set with genomic/molecular character-
ization of brain metastases to date.

In conclusion, despite these limitations, we found enriched expression of a number of novel markers associated with DNA synthesis, replication and repair in brain metastases compared with primary cancer specimens. This trend occurred across tumor histologies, supporting the prioritization of therapies that target these pathways. Although several of the therapeutic target candidates are not brain metastasis-specific genes, overexpressed proteins, or amplified genes, this data informs which targets would be suitable for therapeutic purposes during advanced stage metastasis, with the inclusion of brain metastasis patients.

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Conflict of interest
Amy B. Heimberger serves on the Caris Life Sciences Scientific Advisory Board and is a stockholder in the company. Santosh Kesari serves as a consultant to Caris Life Sciences. Joanne Xiu and Zoran Gatalica are employees of Caris Life Sciences. David Spetzler is President and Chief Scientific Officer of Caris Life Sciences. Sandeep K. Reddy was an employee of Caris Life Science at the time of manuscript preparation. Priscilla K. Brastianos serves as a consultant for Eli Lilly and Angiodynamics and honorarium for Merek and Genentech. Michael A. Davies serves on the advisory board for Novartis, Genentech, Bristol-Meyers Squib, Vaccines, Synder, Sanofi-Aventis and is the PI of grants from Genentech, Bristol-Meyers Squib, Myriad, Merek and Sanofi-Aventis. John F. DeGroot is a consultant for Cellnex, Deciphera Pharmaceuticals, AbbVie, FivePrime Therapeutics, Inc., GW Pharma, Carthera, Eli Lilly, Boston Biomedical Inc, Kairos Venture Investments, Syneos Health and Monteris; serves on the advisory board for Genentech, Cellnex, Foundation Medicine Inc., Novogen, Deciphera, Astrazeneca, Insys Therapeutics, Kadmon, Merek, Eli Lilly; has stock in Ziohpahm Oncology and Gilead; has research support from Sanofi-Aventis, Astrazeneca, EMD-Serono, Eli Lilly, Novartis, Deciphera Pharmaceuticals, Mundipharma; has other financial/material interests in DSMB: VBL Therapeutics, DSMB: Novella, VBI Vaccines Inc. and his spouse is an employee of Ziohpahm Oncology.

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