The Role of Genomic Profiling in Advanced Breast Cancer: The Two Faces of Janus

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Supplementary Issue: Status of Cancer DNA Sequencing in the Clinic

ABSTRACT: Recent advances in genomic technology have led to considerable improvement in our understanding of the molecular basis that underpins breast cancer biology. Through the use of comprehensive whole genome genomic profiling by next-generation sequencing, an unprecedented bulk of data on driver mutations, key genomic rearrangements, and mechanisms on tumor evolution has been generated. These developments have marked the beginning of a new era in oncology called “personalized or precision medicine.” Elucidation of biologic mechanisms that underpin carcinogenic potential and metastatic behavior has led to an inevitable explosion in the development of effective targeted agents, many of which have gained approval over the past decade. Despite energetic efforts and the enormous support gained within the oncology community, there are many obstacles in the clinical implementation of precision medicine. Other than the well-known biologic markers, such as ER and Her-2/neu, no proven predictive marker exists to determine the responsiveness to a certain biologic agent. One of the major issues in this regard is teasing driver mutations among the background noise within the bulk of coexisting passenger mutations. Improving bioinformatics tools through electronic models, enhanced by improved insight into pathway dependency may be the step forward to overcome this problem. Next, is the puzzle on spatial and temporal tumoral heterogeneity, which remains to be solved by ultra-deep sequencing and optimizing liquid biopsy techniques. Finally, there are multiple logistical and financial issues that have to be meticulously tackled in order to optimize the use of “precision medicine” in the real-life setting.

KEYWORDS: breast cancer, molecular alterations, genomic profiling, precision medicine, personalized treatment, tumoral heterogeneity

SUPPLEMENT: Status of Cancer DNA Sequencing in the Clinic

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Introduction

In this review, the latest evidence on genomic alterations that underpin the biology of different subsets of breast cancer, the concept of tumoral heterogeneity as a barrier in precision medicine applications, and the therapeutic implications of these findings are discussed in detail in the context of a case that illustrated the failure to sustain benefit with a personalized treatment approach.

Case Report

A 44-year-old female patient presented with lytic bone lesions in January 2011, while still on goserelin and tamoxifen, two years after completing six cycles of anthracycline- and taxane-based adjuvant chemotherapy and irradiation. A subsequent core-needle biopsy obtained from the skeletal lesion at that time revealed metastatic adenocarcinoma, consistent with ER 100% (+), PR (–), and Her-2/neu (–) breast cancer. Following palliative skeletal irradiation, systemic treatment was changed to anastrazole 1 mg daily following bilateral salpingo-oophorectomy. A new axillary lymph node metastasis was noted 12 months later when the endocrine treatment was replaced with capecitabine at the standard dose and schedule. Nevertheless, a new lesion was seen in the liver along with the reappearance of the axillary lymph node and skeletal progression 18 months after this regimen. Treatment was then switched to exemestane and everolimus, which resulted in stabilization of disease only for three months and several lines of combinations including weekly paclitaxel and bevacizumab; metronomic cyclophosphamide, capecitabine, and fulvestrant; letrozole and palbociclib; vinorelbine; and carboplatin, gemcitabine, and bevacizumab were used subsequently until July 2015, all of which eventually resulted in progression. A repeat core-needle liver biopsy was obtained at that time and submitted for genomic profiling to identify targetable mutations. Comprehensive, next-generation sequencing revealed PIK3R2 G373R-subclonal, PIK3CB E1051K-subclonal, mutations,
and PTEN loss as relevant molecular alterations that could have potential therapeutic implications. With these findings, the patient was referred to a different institution to participate in a phase I trial investigating the role of a PI3K beta inhibitor. Nevertheless, six weeks after initiation of the study drug, the patient returned to our clinic with increasing right upper abdominal pain radiating to the back and significant elevation of liver enzymes. An MRI scan obtained subsequently revealed a serious progression in the liver with extensive metastatic involvement. The patient was immediately placed under eribulin, which resulted in a temporary partial remission initially, followed by progression after six cycles. The patient is currently being treated with weekly nab-paclitaxel.

This case illustrates the failure to obtain a response with two molecular agents that could potentially target the genomic alterations detected in the tumor tissue. Everolimus, which could conceivably be effective in tumors with both phosphatase and tensin homolog (PTEN) and phosphoinositide 3 kinase (PI3K) mutations and the investigational PI3K beta inhibitor, were both unable to sustain a clinical response in this patient who had these somatic mutations. Informed consent was obtained from the patient to use her clinical information as appears in this text.

With the advent of molecular diagnostic techniques, breast cancer has evolved from a single disease into a heterogeneous clinical entity comprised of several different molecular subtypes with distinct biologic and clinical characteristics. Genomic profiling through next-generation sequencing (NGS) has provided an unprecedented pool of data, which has helped to improve our understanding of the biology behind tumor proliferation and metastatic progression. Over the last decade, translational studies from prospective, as well large-scale retrospective trials have led to the identification of a plethora of new breast cancer–associated genes and have unraveled many intracellular pathways associated with enhanced invasive capacity and metastatic potential, as well as resistance to treatment. Furthermore, these comprehensive profiling studies have led to the identification of many genomic alterations that could potentially be targeted by molecular agents, thus resulting in the generation of a new era called “personalized treatment.”

Attempts to enable a more precise prognostic and predictive evaluation by comprehensive molecular characterization and the drawbacks associated with the continuously evolving genomic landscape of cancer per se are discussed in this review.

**Genomic Landscape of Breast Cancer**

Cancer is a disease formed by genetically abnormal cells that have the capacity for uncontrolled growth and evasion of apoptosis. This genomic alteration is accumulated and evolved through the life span of a single normal cell that has survived by adjusting to the microenvironment, escaping immune attack, and developing resistance to treatment. In order to become a “cancerous cell,” a cell has to acquire traits that incorporate oncogenic potential and clonal selective capacity by somatic mutations that occur as a result of erroneous DNA replication or exposure to mutagens and comprise less than 20% of the mutational load of a cancer cell. These mutations are also called “driver mutations.” They are distinct from the more frequent “passenger mutations,” which are biologically neutral and do not confer growth advantage.

Clinically, breast cancer is categorized into three groups based on the response to certain drugs. Hormone responsive subtype comprised “luminal” tumors, which express estrogen and/or progesterone receptors. Endocrine treatment is most likely to be effective in this type of tumor. The Her-2 positive subgroup is a distinct subgroup that displays Her-2/neu overexpression, which results in the activation of epidermal growth factor receptor (EGFR)-related intracellular pathways for cellular proliferation. The development of a receptor-specific monoclonal antibody, namely, “trastuzumab” has changed the natural history of Her-2 (+) metastatic disease with a significant survival benefit. Finally, the triple-negative subgroup, which lacks any of the three receptors, has generated a great deal of attention over the last decade due to lack of specific treatment options other than standard cytotoxic regimens with questionable benefit. Thus, immense efforts have been placed to determine the biologic and genomic mechanisms of metastasis in order to identify targets for effective therapeutic approaches.

Starting from 2012, when the initial report from the Cancer Genome Atlas Network was published, the advent of genomic profiling by NGS has helped unravel the extensive genomic landscape that underlies breast cancer pathogenesis. In addition to the confirmed role of several previously reported somatic driver mutations such as PIK3CA, PTEN, Alpha serine/threonine (AKT1), P53, catherdine 1 (CDH1), trans acting T-cell specific transcription factor GATA3, (Retino-blastoma 1) RB1, mitogen-activated protein kinase3 kinase 1 (MAP3K1, Mixed lineage leukemia 3) MLL3, and cycline-dependent kinase (CDKN1B), many other driver genes that orchestrated the biologic behavior of the molecular subtypes were identified (Table 1).

Translational analyses have led to the classification of breast cancer into four distinct molecular subtypes with diverse genomic signatures: luminal A, luminal B, Her-2 enriched, and basal-like subtype using RNA–sequencing profiles. Luminal A breast cancer is the most abundant clinical subtype and is characterized by hormone responsiveness and expression of genes from luminal epithelium, such as GATA3, Forkhead box protein A1 (FOXA1), and B-cell lymphoma 2 (BCL-2), and lower expression of genes that confer proliferative capacity. Despite the expression of hormone receptors, albeit on a smaller scale, the luminal B subtype is a more aggressive variant with a high level of proliferative gene expression. In luminal breast cancer, the most frequently observed genomic alterations are PIK3CA and TP53, reported to occur in about 40% and 20%, respectively. Somatic mutations in
PIK3CA have been shown to induce oncogenic characteristics by activating AKT. In contrast, inactivating mutations in MAP3 K1 and MAP2 K4, which are mutually exclusive, appear to act by turning down the Jun-N terminal kinase (JNK) signaling pathway. Amplifications in the fibroblast growth factor receptor (FGFR) gene have been linked to a more aggressive phenotype of luminal breast cancer, which is encountered in approximately 10% of breast cancer patients. Finally, there has been a great deal of enthusiasm to identify the role of estrogen receptor 1 (ESR1) mutations, which are linked to resistance to endocrine treatment. These mutations seem to be acquired through the genomic evolution of the cancer cell because the frequency of mutations, which is estimated to be around 5% in primary tumors, increases to approximately 20% in metastatic patients who had previously received aromatase inhibitors. At the genomic level, these mutations seem to result in active estrogen-related signaling in the absence of a ligand. Luminal B breast cancers display a more heterogeneous expression pattern, with TP53 and PIK3CA being the most common, seen in approximately 30% of cases. The inactivation TP53 pathway by alternative intracellular signaling mechanisms such as ataxia telangiectasia mutated (ATM) loss and mouse double minute (MDM2) amplification, as well as epigenetic regulation by turning down the anti-metastatic miR-31 by overexpression of EMSY and Jumonji/ARID domain 1B (JARID1B) genes as luminal lineage-specific oncogenes have been implicated in the relatively aggressive phenotype of this tumor type as compared with the luminal A counterparts.

The Her-2 enriched subtype shows a high expression of growth factor receptor–bound protein 7 (GFRB7), which is located on chromosome 17 in juxtaposition to the Her-2 amplicon. In addition, a high frequency of TP53 (72%) and PI3CA (39%) mutations, as well as a lower frequency of mutations in the PIK3R1 and PTEN gene have been reported. The basal-like subtype is characterized by low expression of hormonal receptors as well as Her-2. At the genomic level, a vast majority of this subtype expresses TP53 mutations (80%), followed by mutations involved in the cytoskeletal functions and oncogenes such as PIK3CA, PTEN, and JARID1B.

### Characterization of Intra-Tumoral Heterogeneity

Studies that focused on full genomic characterization of the primary tumor and their metastases by high throughput molecular techniques have revealed differences in the genomic construction within the same tumor called “intra-tumor heterogeneity”. The term “spatial intra-tumor heterogeneity” refers to the occurrence of genetic aberrations at different geographic locations within the tumor; “temporal intra-tumor heterogeneity” indicates the acquisition of genetic disparities at different time points. The genomic instability of the cancer tissue underpins the continuous dynamic alterations that lead to clonal evolution of the tumor. In the clinical setting, evidence for spatial heterogeneity is provided by reports from various investigators who detected the discordance of ER, PR, and Her-2 expression ranging between 4% and 40% in primary tumors and matched metastatic lesions. Spatial heterogeneity between the primary tumor and its metastatic counterparts at the genomic level has been investigated in a number of studies using different methodologies. The presence of different sub-populations has been shown in a single tumoral mass and has been implicated in the malignant progression of DCIS to invasive carcinoma by clonal selection in a quarter of cases with matched DCIS and invasive cancer tissues. Furthermore, genetic characterization of different lesions from 36 cases with multi-focal tumors revealed that about one-third of the patient dataset did not share mutations in frequently altered genes such as TP53, PIK3CA, PTEN, GATA3, RB1, and FOXA1, despite similar histopathologic features.

Coupled with selective pressure resulting from multiple lines of prior treatments and stromal interactions, distinct genomic subpopulations are selected to form metastatic clones. This phenomenon, which underpins the mechanism by which “temporal intra-tumor heterogeneity” evolves within...
the life span of a tumor, is a major therapeutic challenge in the management of metastatic disease. In their elegant xenograft model constructed from a metastatic basal-like breast cancer, Ding et al.\(^{32}\) showed that metastatic tumors may harbor mutational profiles from minority clones within the primary tumor. In a study that investigated somatic mutational profile of metachronous lesions of a case of lobular ER (+) breast cancer, 60% of mutations detected in the metastatic lesion were not detected within the primary tumor, which suggested a significant molecular evolution within the nine-year interval of metastatic progression.\(^{33}\)

In order to decrease false positivity error rates and capture genes with a low frequency in a heterogeneous cell population, the methodology was fine-tuned to focus on single cells in active division using whole genome and exome sequencing of G2/M phase nuclei called “single cell genomic sequencing” (SCGS). One of the pioneering studies that used this method in two patients with distinct subtypes of breast cancer confirmed the concept of intra-tumoral heterogeneity. In addition, based on the highly similar rate of single-cell copy numbers despite a large number of subclonal and de novo mutations, the authors proposed that although aneuploidy occurs at the onset of cancer evolution, point mutations evolve gradually over time generating extensive clonal diversity followed by the stable expansion of these clones to form the tumoral mass.\(^{34}\) A similar analysis using SCGC on two different basal-like breast cancers showed that each clonal subpopulation within a tumor tissue expressed a distinct molecular characteristic despite sharing highly similar copy number profiles. Further analysis of a phenotypically similar tumor revealed that seeding of a single aneuploid clone from a heterogeneous primary tumor may lead to metastatic progression.\(^{35}\)

### Clinical Applications and Therapeutic Implications

Clearly, the evolution of whole genome sequencing has opened up a new era in the field of oncology. The increasing use of NGS and related molecular methods has provided insight into the biologic basis of cancer development, and also helped decipher the “actionable” genetic codes that could have a potential therapeutic value. Of note, one of the major challenges that lies ahead is the ability to convert this enormous genomic data into clinically useful information that can be used to identify novel targeted agents, as well as overcome resistance to current treatment modalities. First of all, candidate driver genes suggested by the genome-wide assays should be functionally validated by preclinical studies. Then, a translational validation of the mutations and key intracellular pathways suggested by these analyses is required. Finally, actionable genomic data emerging from these validation studies will be integrated to design clinical trials with available matching molecular agents.\(^{36\text{-}38}\) The genomic alterations detected by whole genome sequencing led to proof-of-principle trials in which targeted agents were tested in corresponding molecularly defined clinical series. A major cornerstone has been the identification of Her-2 amplification, seen in approximately 15% of patients with breast cancer, which has been associated with increased cellular proliferation, resistance to standard treatment, and thus poorer outcomes.\(^{37}\) Trastuzumab, a selective monoclonal antibody that targets Her-2, has resulted in a major shift in the natural history of Her-2 (+) breast cancer by providing substantial survival benefit in both advanced and early-disease settings.\(^{38\text{-}41}\) In fact, the success story of trastuzumab, followed by the clinical development of pertuzumab, a monoclonal antibody that prevents Her-2 and Her-3 heterodimerization,\(^{42}\) and TDM-1, a conjugated monoclonal antibody,\(^{43}\) has generated a great deal of enthusiasm for subsequent targeted therapy trials. Nevertheless, despite energetic efforts, only 10–20 actionable driver mutations have been detected in less than a quarter of breast cancer patients to date.

One of the most frequently detected aberrations in breast cancer involves the PI3K/mTOR pathway, which plays a key role in mediating cellular growth, proliferation, and survival, especially in hormone-responsive tumors. Encouraging preclinical data that showed enhanced activity with mTOR inhibitors and anti-estrogen therapy led to clinical trials, which showed significant survival benefit with combined aromatase inhibitor and everolimus treatment.\(^{44}\) Aberrant signaling through the PI3K-AKT-mTOR pathway is one of the main mechanisms that confer endocrine resistance by ligand-independent receptor activation (yeni).\(^{45\text{-}46}\) Not surprisingly, several PI3K inhibitors rapidly completed preclinical development and are being tested in various levels of ongoing clinical trials. Furthermore, palbociclib, an oral highly selective cycline-dependent kinase 4/6 (CDK4/6) inhibitor yielded a significant survival benefit when combined with an aromatase inhibitor in the first-line metastatic setting.\(^{47}\) However, a major problem in this regard is the failure to identify predictive molecular markers because the increasing cost of emerging molecular agents has generated a great deal of debate on the value of cancer care. Despite this, with the exception of Her-2 amplification, none of the aforementioned genomic aberrations has been shown to have a predictive role in defining subgroups that may benefit from their corresponding targeting agent, which suggests that distinct driver-addicted pathways are involved in the carcinogenic evolution of ER (+) breast cancer.

A translational study by Ellis et al.\(^{48}\) confirmed their previous observations that MAP3K mutations were associated with the low-grade luminal A subtype, showing a low proliferation rate; mutations involving the TP53, RB1, and runt-related transcription factor X1 (RUNX1) genes indicated a luminal B subtype with higher grade, rapidly proliferative tumors. The authors suggested that patients who harbored MAP3K or GATA3 mutations may respond to aromatase inhibition, and chemotherapy or different therapeutic choices should be sought for patients with TP53 or RB1 mutations, which are mostly resistant to aromatase inhibitors. In addition, amplifications of FGFR have been linked to a more aggressive biologic behavior.
and endocrine resistance. Based on encouraging preclinical data, several phase I trials with FGFR inhibitors such as lucitabin, dovititin, and nindetabin are underway.49,50

As discussed above, endocrine resistance, which is controlled by certain driver mutations, is a major problem in hormone-responsive breast cancer. It is estimated that about one third of patients display primary or de novo resistance, and approximately 20% of patients acquire mutations associated with secondary hormone resistance.51 There are accumulating data on the role of ESR1 mutations as a driver for molecular alteration in endocrine-resistant luminal breast cancer. Clinical data suggest that patients with acquired ESR1 mutations following treatment with aromatase inhibitors may retain sensitivity to a higher dose of selective estrogen receptor modulators or CDK4/6 inhibition.51–53 These observations have sparked interest in the correct sequencing of subsequent endocrine agents, as well as development of more potent estrogen receptor antagonists. In the near future, we are anticipating more clinical applications involving ESR1 mutations for the management of ER (+) breast cancer.

Despite energetic efforts, no actionable genomic alterations could be identified in triple-negative breast cancer, which led to the lack of targeted therapies with established benefits. Germ-line and somatic mutations in the breast cancer (BRCA) gene are encountered in approximately 20% of patients with basal-like cancer, and these have suggested a possible role for PARP inhibitors, which are undergoing clinical investigation.54 The MAG13-AKT3 fusion gene has been implicated to play an oncogenic role in triple-negative breast cancer.55 Small molecule tyrosine kinases may offer potential benefit by inhibiting the v-AKT murine thymoma viral oncogene homolog-3 (AKT-3) activation generated by this fusion.55 In addition, the increased mutational burden in this tumor type has led to clinical trials with immune check-point inhibitors, which have gained significant popularity in the oncology community over the past couple of years.56,57

Another difficulty that has arisen with the advent of genome-wide analysis is the challenge of making a distinction between driver and passenger mutations. Among the thousands of mutations, pathway activations and epigenetic regulations, detecting the main genomic alteration that underpins the oncogenic evolution and eradicating background noise by identifying bystander pathways is an extremely challenging task. In the TCGA project,2 an integrated pathway algorithm called the “PARADIGM” was used to identify recurrent alterations that were most likely driver mutations. However, this method may miss some low-frequency mutations, which results in failure to detect a potentially targetable genomic alteration. For example, patients with Her-2 mutations, which constitute approximately 2% of patients with advanced breast cancer, may respond to neratinib or other Her-2-targeted therapies.2,58 A method proposed to overcome this limitation is the ultra-deep, single-nucleus sequencing method, which has been discussed elsewhere in this review.

The widespread use of genomic hybridization assays and development of molecular medicine has led to the concept of “pathway-directed treatment” for metastatic disease. Many novel agents are being tested in “basket trials” involving different types of cancers with a similar mutational profile. In a pilot study, von Hoff et al.59 were able to apply matched targeted agents in approximately three quarters of 84 patients evaluated to identify molecular alterations and achieved a longer progression-free survival than the previous standard regimen. In a phase 1 program by the University of Texas MD Anderson Cancer Center, 1144 patients were analyzed for genomic aberrations, among whom 40% were observed to express one or more alterations. Of the 175 patients who were able to receive matched novel agents, there was a significant improvement in response rate (27% vs. 5%, P<0.0001), which translated into a significant survival benefit (13.4 vs. 9 months, P = 0.017) as compared with those treated without matching.60 Nevertheless, the SHIVA trial, which randomized 293 patients with prespecified molecular alterations in the hormone receptor, PI3K or RAF pathways to their corresponding targeted agents or to standard treatment, failed to show a survival benefit with the investigational approach.61

Some groups have focused on clinical trials of mutation-matched targeted agents limited to specific cancer types encountered more frequently because this approach lacks the specificity of different driver mutations that may be overlooked within the plethora of distinct tumor types included. For example, the SAFIR trial included 407 patients with metastatic breast cancer whose tumor biopsies were analyzed for comparative genomic array and DNA sequencing.62 Approximately 70% of patients had sufficient tissue that could be included in the analysis, which yielded 46% targetable mutations; the most frequent of which were PIK3CA (25%), cycline D-1 (CCDN1) (19%), and FGFR1 (13%). Of the 43 patients who could receive a matched targeted agent, clinical responses were seen in 9% and disease stabilization was achieved in 21%. Completed in a very short time period with commendable dedication and energy, this trial provided significant evidence for the difficulty in extending laboratory data to practice daily because only 10% of the patient population who initially enrolled in the study could receive some type of matched molecular agent with only 9% response rate.

**Future Prospects**

Spatial intra-tumoral heterogeneity and the difficulty in predicting the lethal clone pose a major challenge in identifying potentially effective targeted agents. Missing a relevant genomic alteration is always possible because a small biopsy cannot represent the whole tumor, necessitating the need for multiple biopsies from multiple lesions. Furthermore, the constant evolution of tumor and changing targets require subsequent biopsies at each stage of progression, which is not a sustainable practice in the clinic.53,63 It has been shown that mutational profiles obtained from circulating tumor DNA

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shed by cancer cells may provide a noninvasive means to capture information on the genomic evolution of the tumor. Numerous clinical studies are underway to investigate the role of subsequent “liquid biopsies” in providing real-time information on the evolving genomic landscape of metastatic breast cancer.

Given the fact that approximately 20%-30% of patients respond to targeted treatment, more efforts should be placed to elucidate the driver molecular alterations associated with cancer progression. As discussed above, resistance to a given treatment is a major consequence of tumor heterogeneity. Modeling the network of clones acquired during the lifespan of a cancer tissue and integrating epigenetic changes in the carcinogenesis model by the systems approach may lead to a more thorough assessment of the genomic landscape and improve predictive accuracy. In addition, identification of intracellular pathways that regulate tumor-stromal interactions may provide relevant information on the role of receptor binding and immune-regulation for resistance to various cytotoxic and molecular agents. Hopefully, this may lead to the development of a more comprehensive personalized therapeutic approach targeting the intra-cellular molecular alterations as well as regulating the stromal signaling through receptor antagonism and immune regulation.

**Conclusion**

In concordance with the multicenter clinical trials that failed to show a benefit with genome analysis-based treatment decisions, the case presented above provides solid evidence for the strenuous task of precision medicine applications in the treatment of metastatic cancer. The main reasons for the lack of response to two novel genomic-matched targeted agents could be attributed to the intra-tumoral clonal diversity, as well as emergence of resistant clones throughout the carcinogenic lifespan. Furthermore, the alterations detected in the tumor may not necessarily represent the driver mutations underpinning the oncogenic evolution, which led to the omission of the lethal clone in the therapeutic umbrella. In addition, there may be distinct epigenetic and tumor-stromal interactions that may have regulatory roles in disease progression, requiring a more comprehensive strategy that ensures blockade of all bypass escape pathways in order to achieve remission.

The advent of molecular diagnosis has provided deep insight in elucidating the genomic mechanisms associated with carcinogenesis, which has led to a major shift in cancer treatment with the generation of the personalized medicine era. However, the failure to achieve a sustainable and generalized benefit in the clinic stresses the fact that there is more to be done to refine the methodology for a more precise assessment of molecular events associated with cancer progression and resistance. Evidently, close collaboration among scientists, industry, and the clinic is required to develop a more comprehensive personalized therapy approach.

**Author Contributions**

Conceived and designed the experiments: YE. Analyzed the data: YE. Wrote the first draft of the manuscript: YE. Contributed to the writing of the manuscript: YE. Agree with manuscript results and conclusions: YE. Jointly developed the structure and arguments for the paper: YE. Made critical revisions and approved final version: YE. The author reviewed and approved of the final manuscript.

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