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Transforming Growth Factor and the Role of Epigenetic Aberrancies in Oncogenic Amplifications: A New Perspective in Preventive and Therapeutic Arena

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Abstract

Three genetic mechanisms activate oncogenes in human neoplasms: 1) mutations, 2) gene amplification, and 3) chromosome rearrangements. These mechanisms result in either an alteration of protooncogene structure or an increase in protooncogene expression. The role of epigenetic aberrancies in carcinogenesis has been described earlier however to clinicians, the biological implications of epigenetic therapies to prevent cancer and the mechanisms involved have been a mystery. Furthermore, there is no biomarker suggested to track the carcinogenesis steps long before cancer develops, and this has caused a significant lack of proactive and preventive measures to be taken as all recommendations in preventive oncology are either deficiently and blindly made or through screening methods which are too late in the game. Here we explored a very different approach by applying our deepest understanding of epigenetics and carcinogenesis and even further we developed a framework where our clinical findings could translate to the research and vice versa by generating advanced and novel hypotheses on "how we get cancer", by exploring the relation between the host and the tumor cells in a way no one had perceived before. The role of specific cancer stem cell pathways is dissected and how to inhibit each of these initiators using multitargeted epigenetic therapies and off-label medications are explained. We should admit that without considering this sophisticated amazing biological network, cancer will remain an unsolved challenge. Further, we were able to solve this unsolved puzzle by bridging the gap from a hypothetical point of view/hypothesis to possibilities that explain the clinical findings we had observed, and conclude that such an approach can completely change

the way practitioners are treating cancer.

Keywords

Tumor Onco-Promotor, Gene Mutations, Gene Amplifications, Epigenetics Multi Targeted Epigenetic Therapies

1. Background

Centuries later, Abu Ali Sina (Ibne Sina/Avecina), was the first physician to treat a patient with cancer claiming in his book (Cannon of Medicine) that treatment of cancer should not be "harsh" as it makes the cancer "evil". Scientists all around the world just realized that there was something called cancer stem cell which by using cytotoxic chemotherapy (harsh treatments), become more active and cause cancer to recur and become resistant (evil). The old school of thought which was that cancer is an aggressive disease and thereby it requires aggressive treatments is now disputed, only with a price of millions of people dead by the cancer but more impactfully by the treatments itself. By definition, cancer is defined as mutated cells that are driving the tumor growth, and all recent attempts have been to target these mutated cells at best to provide some regression or slow down the tumor from its rapid growth. So far no therapy has been introduced to target cancer stem cells or to focus on the close relationship these cells have with the body itself. In fact, Avecina's model of treatment for cancer focuses the most on the human body as a whole. This strategy is based on acknowledging the fact that tumor becomes rejected as a fetus does in a pregnant mother, by the host if there is no cross talk in favor for the tumor protection and nourishment. The rationale of treating the cause and not the effect best transpires in the concept of epigenetic therapies where the cause of the tumor growth is not the genome mutations rather it is the gene transcription which is completely influenced by the microenvironment. Now we actually know that gene methylation can cause gene mutation and all the genetic tests so far have failed to show what practically speaking mutated genes can be methylated and it has the same effect. The best example is the BRCA gene when it is methylated and it becomes nonfunctional as it is mutated, but the available germline tests only detect the mutated gene and not the methylated gene. Further, we had only known how the microenvironment (the host) could interplay with the existing tumor, and now we are realizing that the host in fact is involved with tumor production/carcinogenesis long before a tumor is created. Here in this article, a breakthrough and novel theory is being explored in which both missed parts of the current practice of oncology (Stem cells and microenvironment) are closely discussed with a mind-blowing discovery in which it suggests that all tumor mutations are secondary to specific growth factor activations, and all tumor gene amplifications are caused or reversed by pure epigenetic mechanisms. Although very few scientists had suggested that gene

amplifications are PRE mutation events, and the fact that general mutagenesis is a side effect of growth with amplification, the exact biological dissection and its correlation with growth factors were never described in the literature. The sequence of events—amplification and mutation may help to explain both the origins of some cancers and the evolution of new genes under natural selection [1] [2]. This is a novel perception and it corroborates with the definition of gene amplification as it pertains to over-expressive transcription, which is exactly what we see with epigenetic transcription of genes. Also, we know that genetic amplifications are indeed early steps in carcinogenesis as are epigenetic abnormalities. Yet cases with very advanced cancer can only be driven by these amplifications and not mutated cells. Gene amplifications also are commonly seen after the use of chemotherapy agents due to tumor-selective advantages and pressure. Please see the literature review section references 2 - 3 below. We discuss a few cases for which this concept was used in their treatment and it corroborated with this revolutionary breakthrough.

In recent years, clinicians have been interested in using liquid biopsy which captures the real-time tumor genomic markup and its relevant tumor burden. The use of these technologies has helped us in the assessment of tumor genomic heterogeneous characteristics and their ability to adapt and switch driver genes at different selective pressure points. For example, we can switch drugs targeting EGFR based on the mutated forms which are evident in liquid biopsy after treatment with the first line. More importantly, we can identify many gene amplifications seen in tumors where the genome stability is somewhat deranged. For example, cases we discuss here can have a mutated gene and several gene amplifications at the same time, or only be driven by gene amplifications. Interestingly regardless of the gene type, the amplifications of the genes were significantly more responsive to the epigenetic therapies. We also had an interesting discovery on the correlation between transforming growth factor levels in the blood and the presence of these tumor amplifications versus mutations. We realized that the levels of TGF-Beta 1 are significantly higher (always higher than the normal range of 6668), if we saw genetic mutations (oncopromoter genes) when the levels are always normal in patients who only carry gene amplifications. We started now to think if there is a correlation between tumor gene mutations and increased TGF-Beta, how does this happen? Does one cause the other? We also faced another question on how can we lower the TGF. The cases we had treated with epigenetic therapies all responded but as we tracked them the disappearing amplifications and response in tumor genetic mutations had different patterns. The amplifications responded without variables, almost one hundred percent of the time. The mutations had a variable response but were still very consistent over time. When looking at the TGF levels in cases with genetic mutations, we had almost reduced or normalization all across the board after epigenetic therapies, but the correlation was not direct. We looked into the literature studying the activation mechanisms of TGF, and we realized that TGF can be induced by the

hedgehog pathway and vice versa. We also realized that when TGF is activated it still has to attach to its receptors, and this is completely under the influence of epigenetic mechanisms mainly ubiquitination. We looked into the effects of compounds we used in epigenetic therapies. One compound, Quercetin had been shown to activate ubiquitination proteasome function on several oncogenes, including Her2. Other medications and compounds, such as Curcumin, resveratrol, and metformin, Avandia have been also suggested in the literature to increase the ubiquitination of TGF-Beta1. Epigenetic therapies containing Quercetin also inhibit the DNMT and can inhibit carcinogenesis through this step too [3].

Further, as we looked into the hedgehog pathway, there had been several compounds that target HH, including Vertarum, and many other teratogens. The drugs we most found interesting were acetazolamide, Ibrutinib, alcohol and nicotin. Interestingly Nicotin despite its carcinogenicity inhibits the cancer cell invasion in ovarian cancer [4] [5] [6] [7]. Also when animals hibernate the HH pathways becomes inactive and cell use of glutamate drops [8] [9]. Such mechanism involved alpha 2 macroglobulin production and inhibition of cartilage transformation to bone (increased hyalorunic acid).

We desired to inhibit all three main cancer stem cell driver genes (Hedgehog, Notch 1 and Wnt) [3]. We already knew that the growth factors (FGF and TGF) are involved in cancer stem cell activation and more importantly the epidero-mesenchymal transition or EMT (NOTCH1 → Wnt → Snail, Slug, Zeb → EMT) as such we designed the treatment protocols that inhibit all targets there.

Epigenetic therapies we used were able to target NOTCH1, Snail and Slug and growth factors. We also hypothesize to target Wnt as well by using Lithium. Specifically, lithium can be considered in cases where there are no tumor mutations by inhibition of GSK-Beta. If epigenetics is blamed for being the cause of all cancers, the hypothesis continues to explain the mechanisms involved (discussed here in section literature review section [8] [10], and therapies should be focused on reversing the cause through the same concept. What triggers epigenetic abnormalities is a different subject of discussion but many factors such as hypoxia have been studied [6]. Oxygen increases the proliferation of stem cells but reduces their life span (from an average of 18 years).

Due to the extensive background work, we preferred to have a literature review section (*a literature review section refers to information that is widely considered as the foundation for the information including terminologies*).

2. Literature Review

1) Three genetic mechanisms activate oncogenes in human neoplasms: a) mutations (such as base substitutions, deletions, and insertions, b) gene amplification, and c) chromosome rearrangements. Chromosomal rearrangements also have been shown to cause gene amplifications in hematological malignancies. These mechanisms result in either an alteration of protooncogene structure or an increase in protooncogene expression. Because neoplasia is a multistep process, more than

one of these mechanisms often contributes to the genesis of human tumors by altering a number of cancer-associated genes. Full expression of the neoplastic phenotype, including the capacity for metastasis, usually involves a combination of protooncogene activation and tumor suppressor gene loss or inactivation. Genetic modifications: include loss or amplification of DNA, loss of heterozygosity (LOH) as well and gene mutations. Gene amplification is a copy number increase of a restricted region of a chromosome arm. It is prevalent in some tumors and is associated with overexpression of the amplified gene(s). Amplified DNA can be organized as extrachromosomal elements, as repeated units at a single locus or scattered throughout the genome. Common chromosomal fragile sites, defects in DNA replication or telomere dysfunction might promote amplification. Some regions of amplification are complex, yet elements of the pattern are reproduced in different tumor types. A genetic basis for amplification is suggested by its relative frequency in some tumor subtypes, and its occurrence in "early" preneoplastic lesions. Clinically, amplification has prognostic and diagnostic usefulness, and is a mechanism of acquired drug resistance [1] [2]. Gene amplification is a typical genetic alteration in cancer, and historically many oncogenes have been identified in the amplified regions. Studies then demonstrated that three protooncogene families—myc, erb B, and *ras*—are amplified in a significant number of human tumors [3]. In this regard, novel cancer-associated genes may remain to be identified in the amplified regions. Recent comprehensive approaches have further revealed that co-amplified genes also contribute to tumorigenesis in concert with known oncogenes in the same amplicons. Do proto-oncogenes become oncogenes (through point mutations or fusions) after amplifications? Considering that cancer develops through the alteration of multiple genes, gene amplification is an effective acceleration machinery to promote tumorigenesis [4].

2) Epigenetic role in carcinogenesis and gee amplification: It is also important to recognize that the conversion of a pre-cancer lesion to a cancer lesion is only possible when there are epigenetic abnormalities in the DNA. For example, when the DNMT (or methyl CpG binding domain/MBDs) are knocked out in the lab, the APC mutated colon CA cells are unable to convert to colon CA. This concept is valid in solid tumors (breast CA, prostate CA, etc.) but not in lymphomas as in this case the tumor cells are possibly committed during the embryol stages of development, as is the case studied for marginal cell lymphomas [4]. It is also known that hypoxia in tumours can influence methylation of the histone H3K9 as well as the chromatin remodeling factors by increasing G9a protein stability and increased EZH2, which hypemethylates H2K4 and 9 [5] We have published this concept in prior literature as well under Epigenetic Tumor Response to Hypoxia: An Epimutation Pattern and a Method of Multi Targeted Epigenetic Therapy (MTET) [6].

3) The Hedgehog pathway has been studied extensively in normal physiological development and fetal growth. The activation of this pathway has been studied since last decade, after scientists realized the close mechanism involved with can-

cer stem cells and plasticity/stemness and this specific target, through different downstream targets such as Wnt and NANOG [11] [12]. Variety of invitro and in vivo studies were performed confirming the importance of this target in different types of cancer, such as pancreatic CA [13] [14] [15]-[29], ovarian CA [30], Gliomas [31]-[40], Brain [41] [42], melanoma [43] [44], prostate [45] [46], esophageal [47], leukemias [48]-[60], medulloblastomas [61] [62] [63] [64], Basal cell carcinoma [65] [66], colon CA [67]-[75]. The variety of tumor cell biology is impacted by the activation of HH, including angiogenesis, stem cell survival, and metastasis [76] [77] [78] [79], and it can promote drug resistance [80]. As such there has been significant interest in inhibition of this target, but the main challenge has continued to be drug resistance which in this case is suggested to be related to SUMO activation. Except for Itraconazole that has shown efficacy on resistant pathways, all other drugs have faced the same challenge in trials [81] [82] [83].

3. Methods and Technology

Multitargeted epigenetic therapy constitutes a combination of DNMT inhibitors from natural sources administered intravenously at certain dosages patented in the United States. This combination includes bioflavones, EGCG, Quercetin and butyric acid. Patients presented here were informed and consented to the therapy and the results were collected and analyzed by an independent party. Each compound was manufactured by prescription of an MD under sterile techniques in FDA-approved facilities. The patient was treated on standards of good clinical practice and a compassionate basis, after obtaining appropriate written consent forms in accordance with regional legislation and principles of the declaration of Helsinki.

The safety of the compounds had been tested in clinical trials at phase I and/or II. Patients received therapies through mediport with sterile techniques and no side effects or toxicities were reported throughout the treatment duration. A total of 19 cases were reviewed from patients who were treated and all had gene amplification identified in their liquid biopsy. 14 had normal normal, or near normal Transforming Growth Factor levels. The average age was 47, and consisted of 15 females and 14 males.

4. Results

The liquid biopsies confirmed the presence of at least one gene amplification, with or without associated gene mutation/copy losses. The most common amplification was reported at CCNE1, and FGFR, followed by EGFR and BRAF amplification. The most common mutations were reported in TP53, Rb1, and PI3KCA, and copy losses at BRCA, ATM, RAD51, PALB2, and Rb1. 14 of 19 patients had normal TGF. The average TGF was 4300 (normal range 867-6662). In the other 5 cases, although had amplifications in their liquid biopsy, (with or without genetic mutations), there were elevated TGF levels. 4/5 of these cases had already

received cytotoxic therapies. Only one case was reported with elevated TGF and genetic amplification (of CCND1) who had not received cytotoxic therapies.

12/14 patients had positive responses in their TGF levels post-therapy that was associated with reduced amplified gene expression in post-therapy liquid biopsies, obtained after 2 - 4 weeks of treatment.

Although there was a clear association between the presence of gene amplifications as the main driver for the tumor growth and elevated TGF, we could not consistently correlate the positive response to the therapy to reduced levels of TGF post-treatment, as such there seem to be other mechanisms involved with the response to the therapy beyond the reduction of TGF.

We review some samples of these cases here.

Case number 1: 40 years old female with a history of invasive ductal carcinoma ER/PR ++ diagnosed in 2016 status post-mastectomy, refused conventional therapies altogether, status post recurrence of her disease in stage four metastasized to the chest wall, ribs, and both lungs, currently seeking alternative therapies for her care.

Her main concern was the pain in the sternum where the large tumor was located. Her initial findings confirmed a germline mutation at SMAR, SNF/SWI.

She reporrtedthat the pain had started to subside in her chest wall, and the mass was less pressing to the sternum after the therapies. Her QOL has improved post-treatments. Her chest discomfort was almost completely gone post-therapy. In exam it shrunk by 50 percent. She was restaged with a PET scan which confirmed stable to improved findings.

Her c DNA reported a reduction of FGFR from 8.5 to 3.5 and other alterations (EGFR and CCNE1 became non-detectable, after 15 days of the trial (measured on 11/29/2021).

Further, her FGFR1 dropped down to 2.5 on 3/11/22, as she continued the care with maintenance IV therapies at a week's schedule. Her CEA also dropped to 16.

On March 10th, she was reevaluated and her Guardant showed complete resolution of CCNE1 and EGFR and a reduction of her FGFR1 down to 2.5 (please see **Figure 1**).

She was restaged on 5/13/22 with a whole body PET scan which showed a partial metabolic response in her large sternum mass (SUV down from 8 to 4.9), interval resolution of left pleural effusion, as well as partial response in her widespread metastatic pulmonary disease; right axillary, internal mammary and hilar lymph adenopathies all responded to the interval therapies. For example, the left posterior medial lung lobe lesion decreased from 3 to 2 cm and activity from 6.2 to 3.4.

She continues to improve with the therapies and significant response manifested in all her markers and scans.

Case number 2: 50 years old female with a history of right invasive ductal carcinoma, in 2010, ER/PR + /Her2 negative, Ki 67 at 30 percent, treated with right

(A0481281)
Patient MRN: N/A | DOB. | Gender: Female
Diagnosis: Breast Carcinoma | Test Number 4

GUARDANT360
Therapy Finder Page

REPORTING

Report Date:	MAR-17-2022
Receipt Date:	MAR-11-2022
Collection Date:	MAR-10-2022
Specimen:	Blood
Status:	FINAL

PHYSICIAN
Mohammad Nezami
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Additional Recipient: N/A

Complete Tumor Response Map on page 2

Summary of Detected Somatic Alterations, Immunotherapy Biomarkers & Associated Treatment Options

KEY Approved in indication | Approved in other indication | Lack of response

Detected Alteration(s) / Biomarker(s)	Associated FDA-approved therapies	Clinical trial availability (see page 4)	% cfDNA or Amplification
FGFR1 Amplification	None	Yes	Low (+)

Additional Biomarkers

Biomarker	Additional Details
Tumor Mutational Burden (TMB)	Not Evaluable
MSI-High	NOT DETECTED

Alterations or biomarkers that were "NOT DETECTED" have been excluded from the summary table above.

We evaluated this sample for 83 genes, including the following guideline-recommended genes for breast cancer

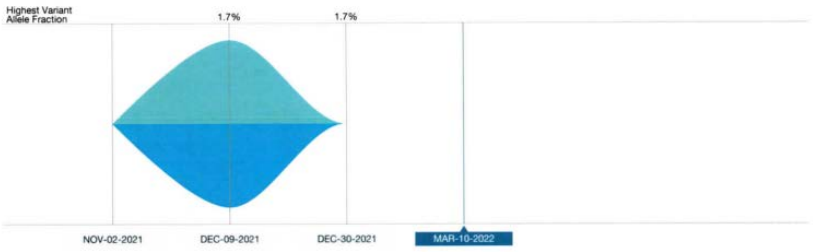
BRCA1/2 | *ERBB2*(HER2) | *PIK3CA*

(A0481281)
DOB: | Test Number 4

GUARDANT360
Tumor Biology Page

Guardant360 Tumor Response Map

The Guardant360 Tumor Response Map illustrates the variant allele fraction (% cfDNA) of observed somatic variants at each sample submission. Amplifications are not plotted, and only the first and last five test dates are plotted. Please see the Physician Portal (portal.guardanthealth.com) for the Tumor Response Map with all test dates.



Detected Alteration(s) / Biomarker(s)	% cfDNA or Amp	Alteration Trend
FGFR1 Amplification Amplifications not graphed above	Low (+)	8.5, 6.7, 6.2, 2.5
CCNE1 Amplification	ND	2.2, 2.2, 2.2, ND
EGFR Amplification	ND	2.2, 2.3, 2.2, ND
ATM Copy Number Loss	ND	1.5, 1.5
BRCA2 Copy Number Loss	ND	1.7, 1.7
NTRK2-FBXO11 Fusion	ND	ND, 0.1%, ND, ND

Plasma Copy Number

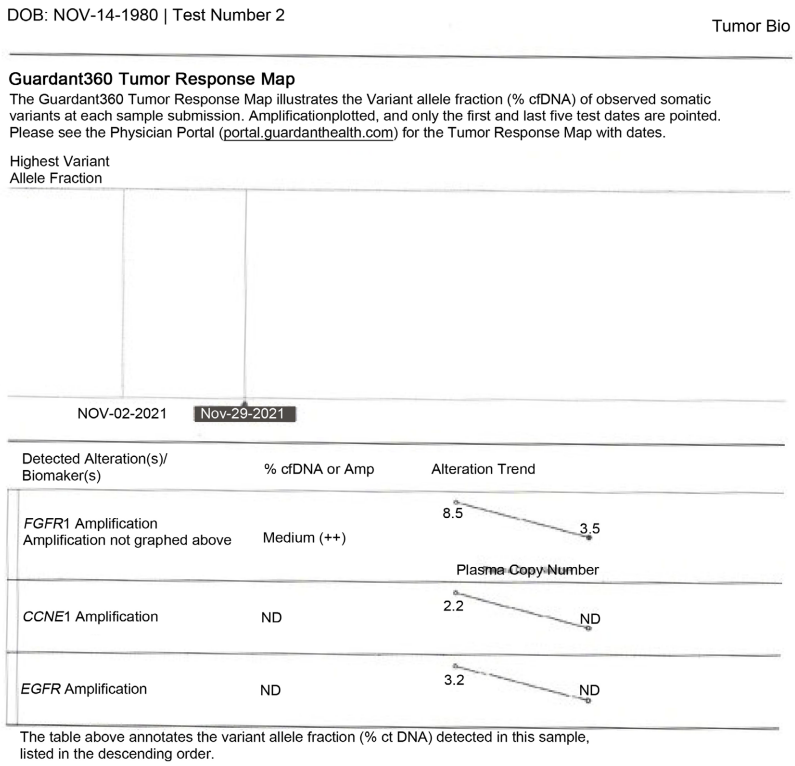


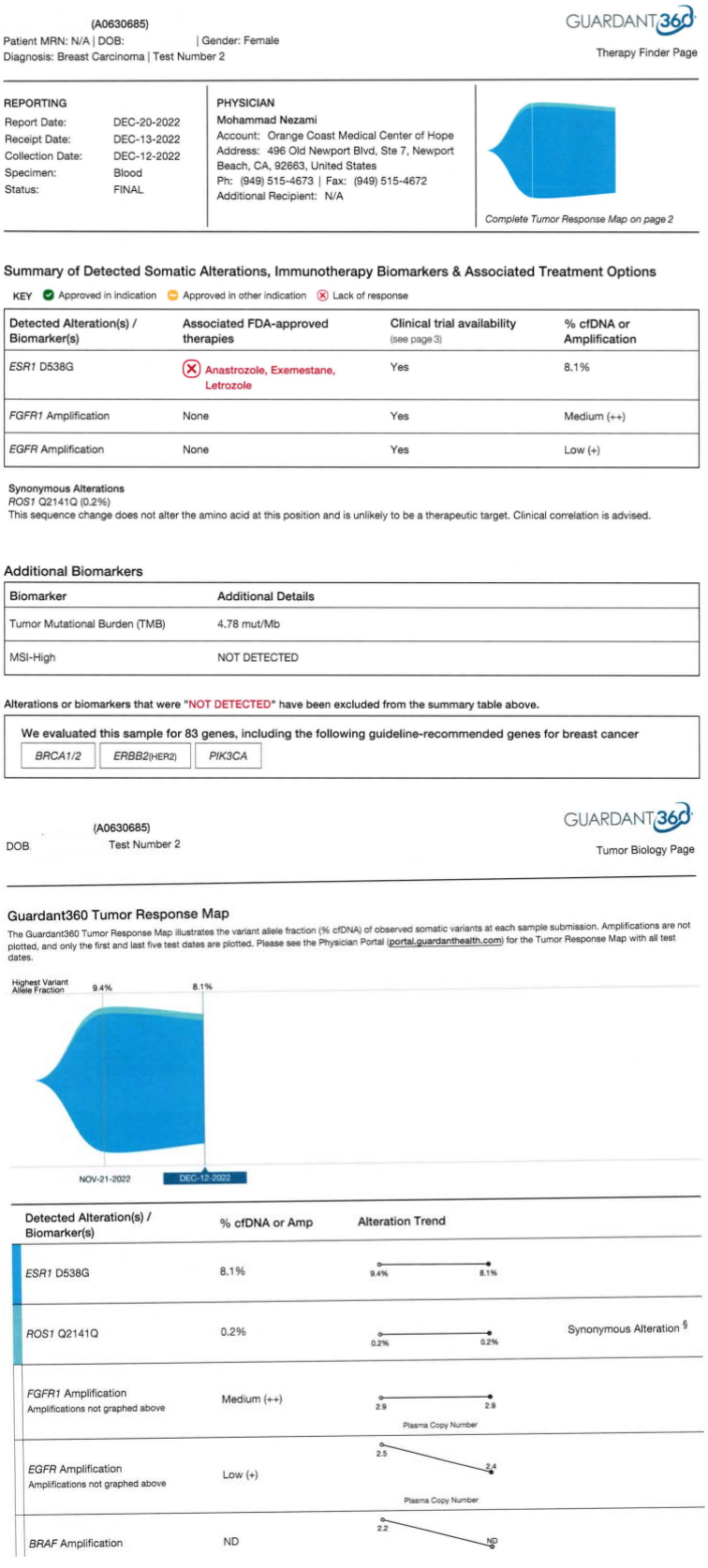
Figure 1. Results of Guardant360 blood test obtained on 11/29/2021 for patient 1 via measured ctDNA from blood samples, showing improvements in all previously detectable amplifications. CCNE1 and EGFR dropped from 2.2 to nondetectable amounts, and FGFR1 amplification dropped from 8.5 down to 3.5.

mastectomy, no hormonal blockade, status post recurrence of disease in 2015, treated with tamoxifen switched to Arometase inhibitors (AI), responded, then Faslodex failed in 2020, started everolimus and examestane and further progressed with liver lesions detected in her scan on August 2022. She had tried some IV vitamin C, poly MVA, tumeric, ozone, mistle toe, and further referred to us seeking a second opinion. The last scan was done on 11/22 which showed liver progressive disease.

Her labs are back and it shows elevated LDH at 319, as well as increased tumor markers, CA 27.29 and CEA at 84. The c DNA results showed EGFR/FGFR/BRAF amplifications and mutated ESR1, the CTC was positive and had very high CK 19 at 114.

Immediately she was started on IV epigenetic therapies which she received on a daily basis. She experienced no side effects from the therapies hiking about 3 miles every weekend. Treatments enhance quality of life. Her labs were repeated after 2 weeks on 12/13/22-Labs showed stable to decreased tumor markers (CEA 82) and LDH (299 and further down to 263), and CA 27.29 dropped from 1856 to 1782, (measured on 11/21 and 12/12/22) but increased liver enzymes (ALK-P and AST/ALT).

Her c DNA results showed reduced MAF of ESR1, EGFR and complete resolution of BRAF ++ amplification (Please see **Figure 2**).



(A0630685)
Patient MRN: N/A | DOB: | Gender: Female
Diagnosis: Breast Carcinoma | Test Number 2

GUARDANT360
Therapy Finder Page

REPORTING

Report Date:	DEC-20-2022
Receipt Date:	DEC-13-2022
Collection Date:	DEC-12-2022
Specimen:	Blood
Status:	FINAL

PHYSICIAN
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Additional Recipient: N/A

Complete Tumor Response Map on page 2

Summary of Detected Somatic Alterations, Immunotherapy Biomarkers & Associated Treatment Options

KEY Approved in indication | Approved in other indication | Lack of response

Detected Alteration(s) / Biomarker(s)	Associated FDA-approved therapies	Clinical trial availability (see page 3)	% cfDNA or Amplification
ESR1 D538G	Anastrozole, Exemestane, Letrozole	Yes	8.1%
FGFR1 Amplification	None	Yes	Medium (++)
EGFR Amplification	None	Yes	Low (+)

Synonymous Alterations
ROS1 Q2141Q (0.2%)
This sequence change does not alter the amino acid at this position and is unlikely to be a therapeutic target. Clinical correlation is advised.

Additional Biomarkers

Biomarker	Additional Details
Tumor Mutational Burden (TMB)	4.78 mut/Mb
MSI-High	NOT DETECTED

Alterations or biomarkers that were "NOT DETECTED" have been excluded from the summary table above.

We evaluated this sample for 83 genes, including the following guideline-recommended genes for breast cancer
BRCA1/2 | *ERBB2*(HER2) | *PIK3CA*

DOB (A0630685) Test Number 2

GUARDANT360
Tumor Biology Page

Guardant360 Tumor Response Map

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Detected Alteration(s) / Biomarker(s)	% cfDNA or Amp	Alteration Trend
ESR1 D538G	8.1%	9.4% → 8.1%
ROS1 Q2141Q	0.2%	0.2% → 0.2% Synonymous Alteration §
FGFR1 Amplification Amplifications not graphed above	Medium (++)	2.9 → 2.9 Plasma Copy Number
EGFR Amplification Amplifications not graphed above	Low (+)	2.5 → 2.4 Plasma Copy Number
BRAF Amplification	ND	2.2 → ND

Figure 2. Reduced MAF of ESR1, EGFR and complete resolution of BRAF ++ amplification.

5. Conclusions

To our knowledge, the influence of epigenetic on gene amplification only has been studied in primitives/yeast and not in cancer, yet the DNA replication machinery is conserved from yeast to humans [11].

Our literature review and further hypothesis helped us to create a protocol to apply to both lower the TGF and inhibit the hedgehog pathway, and correlate the response biologically with clinical outcome. Our findings suggest that all tumor mutations are caused by activation of TGF-Beta 1 exclusively. Tumors that do not produce TGF have no mutations but amplifications, and in these tumors epigenetic therapies can completely inhibit tumor growth when applied to these tumors as evidenced by their liquid biopsy.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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