Heterogeneous Approach for Heterogenous Disease, Heterogeneity in Cancer Genomics and Epigenetic Approach

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Abstract
This article discusses the translational epigenetic science in regulation of the tumor heterogeneity and angiogenesis and addresses the scientific merits of a multi targeted epigenetic therapy (MTET). The critical role of epigenetic therapies targeting the biological behavior of heterogeneous tumors along with related prognostic markers, such as circulatory tumor DNA and circulatory tumor cells, are explored. The relevance of targeting intratumoral hypoxia as a driver for angiogenesis and its correlation with “Genometastasis” and resistance to current available therapies are also discussed. A case series of advanced solid tumors treated with such approach is summarized with future recommendations for clinical trials aimed at modifying the epigenetic signature of the tumor.

Introduction

Hypothesis: This article reviews the current therapeutic challenges related to tumor heterogeneity with specific aim on generating hypothesis from application of epigenetic therapies, in the clinical setting in advanced lung cancer.

Tumor heterogeneity recently described as a variety of distinct genetic and epigenetic profiles and expressions exists in both forms of inter and intratumoral patterns in majority of solid tumors resulting in differences in their morphology and biological behavior. Unfortunately, as much as our understanding of new targets in cancer is evolving, we still face two main challenges: one being unable affect many of the newly described targets, and second being unable to translate a “target response” to a “clinical response”. Current research suggests that the tumors previously treated based on their location, need to be treated based on their behavior, which is mainly under the influence of their genomic signature and its expression. The more we discover about genomic signatures, the more we realize that a simple biopsy of a tumor may not reveal its real “heterogenous” character, as we would need hundreds or even thousands of samples of a single tumor to be able to give the entire picture [1-4]. Newly described strategies have been undertaken to resolve the problems in research, including geographic diffusion molecular profiling. As mentioned, the critical question remains, as we still are not able to pinpoint the driver settings of genes whose alterations have the potential to correlate with survival [4-6]. This problem is further complicated by implementing cytotoxic and targeted therapies, which patients typically receive in a standard setting at the time this paper is published.

The first category, cytotoxic therapies, can cause activation of multiresistance genes, cause mutation in the tumor, and further stimulate the tumors stem cells, which make it more difficult to treat [7]. The second group, targeted therapies, can also cause resistance by inducing mutations in the DNA, as well as enhancing the process called “selective advantage” of the cells that are not responding to the targeted therapy [8]. There is also another phenomenon described in literature called “oncogenic addiction” which is a major problem with even those tumors that are responding, such as is the case with Non-Small Cell Lung Cancer (NSCLC) or Lymphomas treated with targeted therapies [9,10]. This phenomenon is especially problematic when a tumor is more heterogenous, as the tumor may not only become “addicted” to the treatment, where possible flare-ups are seen frequently with cessation of therapy, but also continuation of the treatment causes further selective advantage to the resistant subgroup of the cells which are not responding to the treatment. This is a pattern frequently seen in NSCLC and Breast Cancer where heterogeneity of the tumors is well described [3]. Physicians face a big challenge if they stop the treatment the disease flares-up by the “already treated” cells, and if they continue the same regimen, it increases the suppression of the already treated cells,
giving space to the untreated cells, which will then require further additional therapy. Studies have confirmed that stopping first line targeted therapy “even after its failure” causes increase in tumor size and activity [11]. This example specifically relates to Erlotinib therapy in NSCLC, where that current data suggests continuing the treatment even when a patient has failed to respond [12-14].

To the standard current practice of conventional oncology this is unacceptable, as a physician is asked to continue a therapy when it has already failed, and ask the patient to tolerate the major toxicity involved with these therapies, just because there is no other real option. This dilemma has forced researchers to attempt to develop new treatments including second generation and third generation tyrosine kinase inhibitors (TKIs). These therapies are unable to provide a solution to the cause of the problem: heterogeneity of the tumor. There have been strategies around “sequential tyrosine kinase inhibition” which are intended to reduce the potential and strongly possible effect of secondary mutations in DNA, confirmed by post-therapy circulatory tumor DNA (ctDNA) assessments when a targeted therapy is used. The best example is using the TKIs when pre-treatment circulatory tumor cell (CTC) analysis shows single alteration, and post-treatment CTC analysis shows a wide range of mutations, confirming the fact that the drug itself has promoted heterogeneity of the tumor, on its genomic and epigenomic level [15-18].

The other reason that EGFR blockade is questioned has to do with the fact that EGFR is involved in STAT regulation, which contrary to understanding of many, upregulates the cyclin dependent kinase and has pro-apoptotic effect, as well as increased caspace [19]. This concept was shown in prior studies that showed activation of EGFR could enhance apoptosis. Conversely the inhibition of EGFR can cause resistance to apoptosis, which is why the combination of cytotoxic therapies has failed to improve survival when added to the EGFR blockade in NSCLC in every trial [19-22].

Here we implement a new strategy to use a “heterogenous” approach for a heterogenous tumor. In this approach we have looked in the literature already published in systemic reviews for the mechanisms of resistance in targeted therapies and we have identified following facts:

1) The resistance of a tumor, by selective pressure phenomenon is a common end result of using any and all TKI’s [20-23].

2) The resistance of the tumor correlates with the patients’ survival and is reflected by the circulatory DNA mutational analysis [17,24,25].

3) The strategies of current standard of care to resolve the resistance (such as TKI sequential therapies) has failed, in fact:

The current standard of care induces “heterogeneity” of the tumor, by three mechanisms: first by inducing hypoxia [mainly by cytotoxic therapies] [26], second by DNA mutation and epigenetic alterations of the DNA [18], and thirdly through negative impact on cancer stem cells by increasing their plasticity (increased epithelial-mesenchymal transition, or EMT) [27-29].

To overcome the above problems, we consider following:

We consider using a multi-targeted approach rather than one target therapy. This approach may replace current best standard of care for heterogenous tumors, where the current recommendations are sequential targeted therapy [30]. Instead of using different targeted therapies at different times, we used several targeted therapies at once. This approach has also been suggested by a few researchers in the field. An example was using Erlotinib with Everolimus compared to Erlotinib alone in Lung CA [31-33]. Unfortunately the study failed to show any improved survival. The conclusion was that perhaps the EGFR blockade bypassed the mTOR blockade, or there were other downstream targets that were activated by time, besides the mTOR, such as MAPK kinase. There was also a possibility of activation of feedback loops by using mTOR inhibitors themselves. If this is true, it again confirms our theory that using a targeted therapy can (and most likely will) induce the upstream molecules/genes, as well as parallel transduction pathways [31-33].

The question we attempt to address is which targets need to be modified to be able to minimize the negative impact on tumor resistance while at the same time avoid the oncogenic addiction pattern. We started to look at the upstream targets, rather than downstream targets, as the more a target is downstream, the more likely to create a negative feedback loop [5].

The discovery of feedback loops and the activation of upstream targets pointed again to the reasons for lack of long term survival benefit in most patients when a single target was treated alone. Unfortunately all the upstream targets are still under investigation for development of new drugs, and many of them are not yet approved. In lung cancer it is been suggested that the HSP-70 and -90 are a primary target for both resistance to EGFR blockade as well as ERBB2, Akt, and HIF [34-36]. These drugs yet have not been able to provide an effective tool to overcome resistance in large studies and their high toxicity profile has limited their use. It is worth noting that the combination of targeted therapies such as EGFR and VEGF blockade, in case of lung cancer using Erlotinib and Pazopanib or bevacizumab although exciting in the early stages, [37,38] have also failed to translate in a significantly higher survival in many trials when compared to the standard of care, which is in contrast to our hypothesis. However we explain that this failure due to two reasons, one the fact that the combination therapy is missing to change the tumor’s epigenetic profile, and as discussed earlier negatively impact the genomic stability of the tumor; and secondly the fact that these drugs, although multiaimed in nature, they are not adapted to the mutation allele fraction (MAF) of the tumor, as they are given blindly. Even the combination of these drugs that ultimately blocks all pathways have failed in trials to improve survival, as based on our theory, the ratios for the blockage is more important than the number of targets. In other words, if the tumor is driven by BRAF, and at the same time, has mTOR and KRAS mutations, inhibiting all these targets would perhaps be unnecessary and in fact detrimental as it can cause selective pressure on the other existing cells and activate feedback loops. For example, recent studies have shown that ultimately all colon cancers are RAS positive in a certain ratio, and that targeting EGFR positive cells only increases the growth of KRAS positive minorities [39]. Therefore a multiaimed targeted approach, which consists of targeting the main upstream genes to avoid resistance and activation of upstream genes was initially thought to be reasonable. An example that attempts to overcome this problem is the combination of HSP-70 and -90 inhibitors.
with EGFR blockade. It has been suggested in the literature using Geldanamycin after erlotinib failure in NSCLC may work by inhibiting HSP-90. [40,41]. Another example of targeted therapy failure has been shown in KRAS positive cancers. [42]. Due to frequent mutations in lung, pancreatic, and colon cancer, KRAS offered a good theoretical therapeutic target. Tipifarnib, an inhibitor of farnesyltransferase of RAS protein, failed in clinical trials [43]. BRAF inhibition was not much better. The oral multikinase inhibitor, regorafenib, inhibits BRAF and VEGFR, PD-FR1, FGFR, KIT and RET. Drug resistance, however, occurs early which is supported by loss of function of PTEN and activation of AKT. Additional mechanisms of resistance to BRAF inhibitors include secondary KRAS mutations and activation of MEK/ERK [44]. The MEK inhibitor selumetinib produced encouraging results, but caused resistance through up-regulation of WNT signaling [45].

The end result is that the ratios of the driving mutated cells with actionable targets determine what drugs need to be used. Based on our theory, unnecessarily blocking the targets that are not drivers would enhance resistance.

Therefore:

1) Before using any TKI, (we do not recommend in addition to cytotoxic chemotherapies for reasons explained) we identified the driving mutations. We suggest using ctDNA, as it can guide the possible driving cells through identification of the MAF. Here we could see that assessment of the tumor signature by using circulatory DNA is a useful method, as it can give a snap-shot of the mutations in real time, and also provides a prognostic value for the therapy’s effectiveness. Based on our work, the tumor targets are identified, their ratio of penetrance calculated, and the therapy designed to have a real effect on the most relevant cells with minimum effect on the feedback loops. Using the minimum dosage of the targeted drug, (for example in NSCLC with EGFR positivity) we have shown using 1/16 of the standard dosage is effective. The rationale is that the anti-EMT response and CSC response by TKIs are NOT dose dependent. We use targeted therapies at this low dose only as a measure to reduce oncogenic addiction, keeping in mind that we avoid inducing tumor selective pressure at all times. Epigenetic therapies are indeed extremely important to achieve this. In the case for EGFR blockade, we now know that the EGFR target when methylated does not respond to the EGFR blockade and therefore epigenetic demethylation of EGFR is vital to the response to EGFR TKI [46] and prolongs the response much beyond the estimated 12 months in the trials. In fact we reverse the potential failure of such drugs substantially.

2) Second we attempted to the tumor’s heterogenous features by modifying its behavior. We were especially interested in the targets that are involved with the heterogeneity of the tumor. We found that more hypoxic tumors disseminate more ctDNA [47,48]. The more hypoxic a tumor, the more heterogeneity is seen. Therefore we were interested in regulating the intratumoral hypoxia through epigenetics. In concert with this we identified a major target: HIF-1 and 2, and we designed the MTET therapy around the epigenetic regulation of HIF. Here we have shown in vitro studies to prove our concept by looking at the HIF and tumor integrity in 3-dimensional culture media, and proving the effectiveness of the MTET over HIF related pathways and response elements.

By changing the tumor’s behavior, by reducing the tendency for "genometastasis" [49,50] and dissemination of the tumor’s signature through CTC and ctDNA, we reduce the tumor’s ability to metastasize. It is well studied in cardiovascular research that the cellular dissemination of c DNA is a trigger for stem cell plasticity and inhibits the differentiation of such cells to adipocytes [16]. This process is related to the activation of TLR 7 and 9 (Toll-like receptor 7 and 9) which occurs when the c DNA is globally hypomethylated [51]. The c DNA that is aberrantly methylated can mimic pathogens, and is recognized by the immune system as a target for activation and further catastrophe of immune related cancer stem cell activation by microenvironmental influence. It has also been shown that the hypomethylated (or CpG hypermethylated) DNA can penetrate into the normal distant cells and act as a virus to infect the normal cells as a new host for the cancer, which could then colonize into a new metastatic lesion [50,51].

It is mentioned earlier that our approach based on new understanding of cancer biology is revolutionary as it contradicts current standard of care, which is more a phenotypical therapy, as we know all cytotoxic therapies and majority of targeted therapies increase the heterogeneity of the tumor and its behavior. Studies have confirmed that presence of ctDNA alternates with the use of cytotoxic drugs, yet can be used as a marker for response to the therapy [52]. All trials so far (such as S0500) have failed to prove a strategy around targeting the CTC by switching the chemotherapy regimens in breast cancer for example [53]. The rationale behind this certainly is related to increased epithelial-mesenchymal transition and hypoxia related expressive genes after cytotoxic therapies in general.

The failure of the current targeted therapies may have to do with the non-adaptiveness of such a method, where the tumor genetic instability causes a very dynamically transforming signature that can only be effectively treated if this instability is reduced (by epigenetic therapy). It has been suggested that targeting heterogeneity at the epigenomic level represents an effective strategy in the treatment of cancer and monitored by its signature (DNA methylation) in ctDNA [51,54] as emphasis on the pattern of system evolution rather than specific pathways provides a global and more meaningful approach. This is also important in relation to current subject of discussion for tyrosine kinase resistance. The rapid kinetics, the reversibility of acquired drug resistance and the absence of genetic mutations suggest an epigenetic basis for drug sensitivity. Therefore the multi targeted approach should also target the epigenetic targets, to enhance stem cell differentiation and reduce the heterogeneity as well as drug resistance. This plasticity has been described [55] as required for evolution of cancer stem cells during tumorogenesis that can involve movement between cell populations in a reversible fashion. Epigenetic plasticity could impact altered genetic expressions, in the creation of cellular heterogeneity in cancers of all types. Further a reversed epigenetic modification that can correct the aberrancies may reduce the “stemness” of such multipotent cells to a more defined clonogenicity, and lead to reduction in dynamic heterogeneous tumor cell population.
state. Such genomic architecture changes are modifiable even after genetic and epigenetic mutations have occurred. It is also important to know that heterogeneity is also seen in epigenetic signature of tumors.

**Results and Discussion**

Here we review thirteen (13) cases of NSCLC and review their outcome. 12 patients with the diagnosis of Stage IV lung adenocarcinoma, and one case of squamous cell carcinoma, all refractory to the current standard of care. We will discuss in detail three cases of lung adenocarcinoma with and without EGFR positive status resistant to standard of care, who we treated successfully with this approach, known as Multitargeted Epigenetic Therapy (MTET). Each patient received MTET therapy per protocol, which is a combination of natural polyphenols injected intravenously. These natural polyphenols have a synergistic effects, and when given in combination, target epigenetic marks such as DNA methylation and histone deacetylation. Therapeutic dosages were customized for each patient, based on factors such as drugs metabolism (after testing of CYP450 and other CYP enzymes), as well as body weight. Our evaluation of Natural Killer cell activity as a marker for immune function did not correlate with therapy related response.

The clinical findings and results were far beyond the expectation in majority of the cases. Most of these patients did not receive any concurrent chemotherapy, except in three cases, where palliative chemotherapy was combined with their treatment. We followed the response through the discussed prognostic markers for survival and following results were obtained:

1. Improved quality of life, 12/13 patients had improved quality of life based on their function and ECOG score. One patient who had no change in ECOG was receiving chemotherapy.

2. Improved plasma VEGF/IL-8: All cases with increased angiogenesis markers responded to the treatment with reduced plasma VEGF/IL-8. The magnitude of response was parallel to the reduction of the tumor markers. There were nine cases with increased VEGF and all dropped to normal levels in a quick fashion, over two weeks of treatment.

3. Improved imaging: Improved imaging was seen in Two cases with brain metastasis, one, refractory to multiple sessions of radiation responded with imaging confirming stable disease after three months under therapy.

4. Improved tumor markers (CYFRA 21.1, FGF-2, CEA): All cases with increased tumor markers (ten cases) responded to the treatment, with significant reduction of the tumor markers. Average reduction was noticed in 2 weeks of therapy.

5. Improved circulatory tumor cells/circulatory tumor DNA: all cases with positive CTC or ctDNA responded to the treatments, with complete disappearance of the CTC from the blood. The CTC samples were positive for Histone deacetylase and DNA methyl transferase overexpression, as well as CK19, telomerase and C MYC. Samples were negative post-therapy. Median duration of treatments was 10 days. Circulatory tumor DNA in two cases were obtained, and showed positive mutations in PS3/KRAS/ROS1 and cMET. The tests were repeated after 21 days and showed reduction of the MAF on the altered genes. In one case the KRAS ctDNA decreased, in the other case the ctDNA completely resolved. The altered marker was BRCA2, which disappeared post therapy. Duration of therapy was 16 days. None of these cases received chemotherapy.

6. Overall Survival: Three cases that received concurrent cytotoxic chemotherapy had poor outcome. They passed away after sessions of therapy or could not continue the palliative chemotherapy due to the side effects. One patient who stopped the treatment also passed away in less than 12 months, as was treated with a targeted therapy. The rest of the patients (nine cases) lived beyond their expected life expectancy. Out of 10 patients with Stage IV refractory disease, only one died in less than 12 months. 90 percent of treated patients, who continued care, lived beyond expected life expectancy. 6 patients are still alive 3 to 7 years after their diagnosis and treatment of Stage IV lung cancer.

**Case 1**

57 year old female with oligo-metastatic Non-Small Cell Lung Cancer, diagnosed in 2013, status post-surgery (right middle lobectomy), radiation for stage one adenocarcinoma (T1, N0, M0), Status post recurrence with solitary pleural base nodule in September 2013, and status post stereotactic radiation to a paraspinal mass and chemotherapy consisting of four cycles of Carboplatin and Alimta given from November 2013 to January 2014, with progression of disease.

The markers which were positive before starting the treatments were negative after therapy. cMYC and Histone deacetylase markers were normalized. This proves that MET therapy targets include cMYC and HDAC (Case 1).

The circulatory tumor cell analysis was repeated on 4/9/15 after missing two weeks of treatment, and the results show that although the cMYC was still negative, the CTC appeared with lower expression of CK19, and it was concluded that the tumor cell burden in the blood was low. These results prove that the epigenetic therapies were able to maintain the negative cMYC after two weeks, however the dissemination of CTCs would appear with less intensity and tumor burden after two weeks of cessation of treatments. It was also noticeable that the presented CTCs in the blood sample did not have overexpression of EGFR, confirming that the response was not related to the prior treatment with Erlotinib. The patient started the treatments again on a daily basis. On 6/9/15 the PET/CT was repeated and showed resolution of previously described prevascular node, as well as pulmonary nodule in the posterior right lobe, with minimum residual disease at SUV activity of 1.2. All pulmonary nodules have no FDG activity and there was complete resolution of hilar mediastinal, axillary, and supraclavicular lymph nodes previously described as active.

**Case 2**

69 year old male with history of stage 3B adenocarcinoma of the lung diagnosed initially in March 2015 after three months of undiagnosed cough. He was treated for tuberculosis and Cryptococcal infection, which were found in bronchoscopy. Malignancy then was identified after a CT guided biopsy of the lung mass, which revealed KRAS/ALK/EGFR negative tumor, with contralateral supraclavicular lymph node. After a month, the patient underwent a brain MRI, which revealed a 4 mm brain mass in the parietal lobe, with associated edema.
He was fatigued and complained of cough and was taking TB medications for a latent TB. He also had history of COPD/HTN/CAD/DM/hyperlipidemia.

The patient was started on IV epigenetic therapies immediately following the initial labs that confirmed he had increased neuron specific enolase and CEA. He received 5 treatments and his labs were repeated which showed improved CEA and LDH. Initial CEA was 68.6 and improved to 55.9, while LDH was 323 and decreased to 306. The patient did not change his diet, nor did he receive any other therapies. His quality of life improved. His labs were repeated after 18 sessions of treatment, which showed substantial reduction in neuron specific enolase from 13.8 on 5/04/15 and 16.4 on 5/11/15, down to 9.4 measured on 6/12/15. His LDH stabilized at 304 and his ESR dropped from 90 to 58. His VEGF was reported at 176 on 5/11/15, and repeated post therapy on 6/12/15 and had dropped to 60. On 6/22/15 his brain MRI was repeated with fusion stereotactic resolution (MR spectrum) and showed no change in the size of the tumor, indicating stable disease. His FGF-2 was repeated after 18 treatments, which dropped from 47 down to 20 (dated 5/21/15 and 6/12/15, respectively). This response was obtained without any chemotherapy.

This is the first time a report of a stabilized malignant adenocarcinoma metastasized to the brain treated with epigenetic therapy without any other treatments (Case 2).

Case 3

65 year old female with history of Non-Small Cell Lung Cancer diagnosed in 2007, status post right lower lobe lobectomy. Patient underwent several rounds of chemotherapies consisting of 6 rounds of carboplatin and alimata, completed in July 2013, with good response. Patient was status post recurrence documented in January with new lesion in T12, status post four injections of zometa and several rounds (40 sessions) of TNF enhancing therapies (Immunopheresis) administered by Dr Lentz in Germany. Patient had radiation to the T12 lesion in February 2013, as well as Sutent taken for 27 days and discontinued due to severe side effects in June 2013. The patient was referred to us for evaluation and IV epigenetic treatments in March 2014, and was started immediately after lab were drawn, which showed increased CEA (as a prognostic marker for NSCLC) which was 17.9, dropped to 17.0 after four treatments, received over one week. The patient’s FGF-2 was also checked, which showed response to treatment when it dropped to 15.8 from 18.9, measured on 3/12 and 4/1/14 respectively. It further dropped to 4.7 on 4/23/14 after three more weeks of treatment. The patient had a restaging PET scan on 5/20/14, which confirmed decreased metabolic activity of the tumor in all thoracic and chest lesions. The abdomen and pelvis had stable disease, with maximum SUV activity reported at 4.0 from gastrohepatic ligament (from 3.3). There was an unchanged T12, bone lesions in thoracic spine, compared to prior PET. The supraclavicular (5.77 to 3.7, and 3.1 to 2.2), as well as hilar (3.3 from 3.5, and 2.9 from 4.1), mediastinal (4.3 from 7.6) and paratracheal thoracic LNs (4.6 to 2.1) all responded to the treatment, with decreased posterior cervical completely resolved. Left pleural thickening and nodule completely disappeared.

Further she continued the treatments at our clinic, with documented stable to improved disease in her restaging scans. All her thoracic disease improved to almost no significantly FDG avid disease and her peritoneal metastasis was stable to improved.

This represents a positive response in a patient with stage four disease to Epigenetic treatment, refractory to chemotherapy/and immune therapy. This patient continues our therapy with no reported toxicity on maintenance program.

Conclusion

The clinical application related to the scientific rationale discussed in this article provided clinical benefit beyond expected in traditional setting defined by historical and comparable data in a subset of patients with heterogeneous disease. Further hypothesis can be generated based on these preliminary findings, aiming at improving patients’ survival in refractory and heterogenous disease.

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4. Megan Elam, Research Coordinator – Pacific Medical Center of Hope

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Case 1

Analysis Report of Lab No. 4401571854 from 15.01.2015

Patient: Lung CA, NSCLC

For the analysis, we performed the following work steps

1. Isolation of circulating tumor cells / micrometastases

   In order to obtain circulating tumor cells from the patient's peripheral blood, epithelial cells were isolated. A preparation of mononuclear cells (MNC) served as a control cell fraction. From all fractions mRNA was isolated. Afterwards, the expression of tumor-relevant genes was measured by quantitative real-time RT-PCR.

2. Molecular detection of circulating tumor cells

   The following molecular markers were used to detect tumor cells:

   - **Telomerase**: The expression of the telomerase-gene can be increased in most tumor types, but not in normal tissue. An increased expression of the telomerase gene may be indicative for the presence of tumor cells in the circulation.
     - neg: Expression of telomerase was not detected in the isolated cells.

   - **C-MYC**: Overexpression of C-MYC indicates an increased proliferation-rate of the isolated cells. An increased proliferation-rate is a typical feature of tumor cells.
     - **pos**: The expression level of C-MYC was elevated.

   - **ERBB2**: Overexpression of ERBB2 (HER2/NEU) is a trait of different types of cancers and may be observed also in lung cancer. Thus, the detection of ERBB2 overexpression may be indicative for the presence of circulating tumor cells.
     - neg: Expression of ERBB2 was not elevated.

   - **CK19**: The detection of an expression of the cytokeratin 19 (CK19) gene indicates the presence of cells of epithelial origin and is thus indicative of circulating tumor cells.
     - neg: There was no expression of CK19 detected.

   - **HDAC1**: aberrant expression of HDAC1 (histone deacetylase 1) has been described in various human tumors, including lung cancer. Overexpression of HDAC1 can therefore serve as a molecular biomarker for tumor cells.
     - **pos**: HDAC1 overexpression was detected

Interpretation

In the isolated tumor cell fraction, elevated expression of C-MYC and HDAC1 was observed. This finding may indicate the presence of circulating tumor cells in the analyzed blood sample.
Analysis Report of Lab No. 4401571888 from 05.02.2015

Patient: Lung CA, NSCLC

For the analysis, we performed the following work steps

1. Isolation of circulating tumor cells / micrometastases
   In order to obtain circulating tumor cells from the patient’s peripheral blood, epithelial cells were isolated. A preparation of mononuclear cells (MNC) served as a control cell fraction. From all fractions mRNA was isolated. Afterwards, the expression of tumor-relevant genes was measured by quantitative real-time RT-PCR.

2. Molecular detection of circulating tumor cells
   The following molecular markers were used to detect tumor cells:

   **Telomerase**
   The expression of the telomerase-gene can be increased in most tumor types, but not in normal tissue. An increased expression of the telomerase gene may be indicative for the presence of tumor cells in the circulation.
   neg: Expression of telomerase was not detected in the isolated cells.

   **C-MYC**
   Overexpression of C-MYC indicates an increased proliferation-rate of the isolated cells. An increased proliferation-rate is a typical feature of tumor cells.
   neg: The expression level of C-MYC was not elevated.

   **ERBB2**
   Overexpression of ERBB2 (HER2/NEU) is a trait of different types of cancers and may be observed also in lung cancer. Thus, the detection of ERBB2 overexpression may be indicative for the presence of circulating tumor cells.
   neg: Expression of ERBB2 was not detected.

   **CK19**
   The detection of an expression of the cytokeratin 19 (CK19) gene indicates the presence of cells of epithelial origin and is thus indicative of circulating tumor cells.
   neg: There was no expression of CK19 detected.

**Interpretation**
In the fraction of isolated tumor cells, abnormal expression of all measured tumor-associated marker-genes was not observed.

**Conclusion**
According to the panel of molecular tumor markers used for this analysis, there are no indications for presence of cancerous cells in the analyzed blood specimen.
Analysis Report of Lab No. 4401690305 from 09.04.2015
Patient: Lung CA, NSCLC

For the analysis, we performed the following work steps

1. Isolation of circulating tumor cells / micrometastases

In order to obtain circulating tumor cells from the patient's peripheral blood, epithelial cells were isolated. A preparation of mononuclear cells (MNC) served as a control cell fraction. From all fractions mRNA was isolated. Afterwards, the expression of tumor-relevant genes was measured by quantitative real-time RT-PCR.

2. Molecular detection of circulating tumor cells

The following molecular markers were used to detect tumor cells:

- **Telomerase**: The expression of the telomerase-gene can be increased in most tumor types, but not in normal tissue. An increased expression of the telomerase gene may be indicative for the presence of tumor cells in the circulation.
  - neg: Expression of telomerase was not detected in the isolated cells.

- **C-MYC**: Overexpression of C-MYC indicates an increased proliferation-rate of the isolated cells. An increased proliferation-rate is a typical feature of tumor cells.
  - neg: The expression level of C-MYC was not elevated.

- **ERBB2**: Overexpression of ERBB2 (HER2/NEU) is a trait of different types of cancers and may be observed also in lung cancer. Thus, the detection of ERBB2 overexpression may be indicative for the presence of circulating tumor cells.
  - neg: Expression of ERBB2 was not elevated.

- **CK19**: The detection of an expression of the cytokeratin 19 (CK19) gene indicates the presence of cells of epithelial origin and is thus indicative of circulating tumor cells.
  - pos: There was weak expression of CK19 detected.

**Interpretation**

In the isolated tumor cell fraction, expression of CK19 was observed. This finding may indicate the presence of circulating tumor cells in the analysed blood sample. Since CK19 was only slightly elevated and all other detection markers had been negative, the tumor cell burden in blood is probably low.
Case 2

Guardant360 Tumor Response Map

The Guardant360 Tumor Response Map illustrates the relative changes of observed ctDNA at different sample submission time points. The "Somatic Alteration Burden" value below refers to the maximum % of ctDNA detected at each time point. Amplifications are not plotted.

Summary of Alterations & Associated Treatment Options

The percentage, or allele frequency, of altered cell-free DNA (% of ctDNA) circulating in blood is related to the unique tumor biology of this patient. Factors that may affect the amount/percentages of detected genomic alterations in circulating cell-free DNA in blood include tumor growth, turn-over, size, heterogeneity, vascularity, disease progression, or treatment.

<table>
<thead>
<tr>
<th>Alteration</th>
<th>Mutation Trend</th>
<th>% of ctDNA</th>
<th>ctDNA Amplification</th>
<th>FDA Approved in Indication</th>
<th>Available for Use in Other Indications</th>
<th>Clinical Drug Trials</th>
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<td>H2014R</td>
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<td></td>
<td>None</td>
<td>None</td>
<td>Trials Available</td>
</tr>
<tr>
<td>KRAS</td>
<td>G12S</td>
<td>6.7</td>
<td></td>
<td>None</td>
<td>Trametinib</td>
<td>Trials Available</td>
</tr>
<tr>
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<td>A1921S</td>
<td>2.7</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>MET</td>
<td>L515L</td>
<td>0.2</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

For a more detailed Guardant360 Patient Report, log onto: https://portal.guardianhealth.com
To set up an account, contact Client Services: 855-985-0888.
The chart above annotates the percentage or allele frequency, of altered circulating cell-free DNA (% of ctDNA) detected in this patient. The detected genomic alterations are listed in descending order by % of ctDNA by gene.

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