Epigenetic therapies, immune therapies, and anti-angiogenic therapies: The triad of oncology. 

Angiogenesis: Cause or effect?

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Advances in technology have allowed medical providers to customize healthcare with medical decisions, practices, and therapeutics being tailored to the individual patient. Through the use of advanced laboratory testing, we are better able to understand the dynamic and heterogeneous nature of cancer. One of the challenges oncologists and practitioners face in the treatment of cancer is the clinical application of targeted therapies, when a tumor consists of a variety of genomic signatures. Laboratory studies identifying markers for angiogenesis, circulating tumor cells, and circulating tumor DNA have allowed the opportunity for the development of treatment plans tailored to a patient’s individual genetic and epigenetic profile. Understanding the genomic and epigenomic signature of a tumor advances the practice of precision medicine, leading to improved response rates and improved overall survival.

The dependence of tumor growth on the development of a neovasculture has been well described in the literature. One of the best studied angiogenic factors is vascular endothelial growth factor (VEGF). VEGF belongs to a family of homodimeric glycoproteins that bind to three different VEGF receptor tyrosine kinases in an overlapping pattern. They share the regulatory mechanisms with other growth factors, such as PDGF. These include receptor dimerization and activation of tyrosine kinase, as well as creation of docking sites for signal transduction, which direct migration, survival, and proliferation.

Angiogenic inhibitors are superior to immune therapies, and have replaced the current standard of care in majority of solid tumors, where tumor migration and metastasis are the main indicator of the patients’ survival. Although there have been great efforts to inhibit angiogenesis by using targeted therapies, recent investigations have failed in the majority of clinical trials using VEGF inhibitors, shifting current efforts back to immune therapies. The mystery still remains, as all the pre-clinical findings in the laboratory contradict the human data, pointing to the interference of human stroma in relation to failure of such therapies in clinical trials. [1,2,3,4] The more we study this interference, the more it has become apparent that angiogenesis is a pattern of events that is supported by the stroma, and that although angiogenesis increases the tumor’s access to vessels and further metastasis, it reduces the tendency of a tumor to activate potential growth related factors beyond VEGF. [5,6,7,8] In other words, loss of vessels as a result of using angiogenesis inhibitors in a tumor will activate the production of growth related factors, (such as PDGF, EPO, MET and TGF-alpha) as a result of induced hypoxia, and further enhance the tumor growth and aggressiveness.[9,10,11]
Many efforts in destroying the stroma’s support in tumor growth have also failed to show a positive response. As hypoxia is induced in every trial where the stroma is the target of therapy. For example, in pancreatic cancer, pre-clinical studies showed enhanced tumor growth when the fibroblasts around the tumor were targeted by antibodies. We have also identified HSP-70 as a target to induce HIF, which can be triggered by stroma. Finally, we have learned that there are negative feed-back loops that become activated by blocking VEGF, which increase the activation of PI3K, through IGF-1 and PDGF. This alone can be a disastrous event in the long term, as it will break the temporary Band-Aid of blocking VEGF by targeted therapy. This phenomenon is even true in the case of the most promising therapies, such as m-TOR inhibitors, where we initially thought that m-TOR inhibition indirectly blocks HIF, and directly blocks the VEGF production. Further developments of multi-targets related to VEGF (VEGF Trap) have also failed to show clinical efficacy.

Immune therapies in general have also shown a similar outcome, which is a lack of sustained and durable response in many types of cancer. The temporary gain is sometimes exciting. Due to the mechanisms described here, this method has also failed to present an attractive approach, as the initial gain does not translate into overall survival in the majority of solid tumors. Again, the interaction of the stroma is a common reason for failure. Checkpoint inhibitors, such as PD-1 inhibitors and TNF-receptor blockers, as well as other means of immune therapies, have been shown to cause stimulation of NF kappa-B pathway, and almost 150 related onco-promoter genes. These have been shown to be the most significant negative side effects, causing activated down-stream pathways. Secondly, they stimulate cancer stem cells, which translates to recurrence of disease and poor clinical outcome and prognosis. An example is the use of IL-2 in renal cell carcinoma, which shows an exciting durable response, and still absolutely no survival benefit. When IL-2 was further investigated, there were several other genes identified to be triggered, including Cyclin D-1 and MEK/ERK/RAF pathway. Also, STAT3, and other genes notorious for oncogenesis were activated in the long term, causing tumor aggressiveness. As a result, there was no change in survival, even in the settings of tumors where these therapies were known to be the first line treatment (such as renal cell carcinoma). This lack of advantage was also seen when the researchers tried to combine immune therapies with angiogenic inhibitors (such as the case for Avastin combination with Interferon-alpha in RCC). The rationale behind such combination is related to the fact that TNF-alpha and related factors increase angiogenesis (and can cause significant vascular leak syndrome), as well as FGF, hepatocyte growth factor, VEGF, and TGF, all of which can be measured in patients' serum, as markers for angiogenesis. It is evident that VEGF has immune suppressive effects on its own.

Recently, epigenetic therapies have become an exciting area of research after some preliminary reports showed improved survival in patients with hematological disease. However, these therapies were mostly unselective for DNA targets. The complexity of the epigenome, and more specifically the cancer stem cell targets, made this category of drugs extremely difficult to be tuned to the exact number of the targets to be effective, and at the same time, not be harmful to the normal stem cell function. Many scientists began to look into using epigenetic coding and sequencing in order to overcome this barrier, however, it was later observed that the epigenetic aberrancies are a result of a dynamic process, and any effort of therapy has to become adaptive to such dynamic process. As clinicians and researchers focused on a quick fix for a problem that had been accumulated over decades, they lost interest, as many of these therapies did not improve survival. Examples include using butyric acid in gliomas and multiple myeloma trials.

As the result of exhausting all these therapies, we have established the pitfalls in these approaches. Every time we failed in a chess game, we learned more about the complexity and sophisticated nature of the cancer cell and its biology.

Here, we propose a “road map” that reflects the lessons we have learned. This road map recognizes the interactions of angiogenesis with immune system and epigenetics, and re-emphasizes the interaction of stroma. It is also our aim to “refine” the goals of research and clinical trials of the future, by stepping back in our efforts to eradicate the cancer cells, and instead modify the tumor’s behavior.

**Methods**

1. We suggest that we redefine the definition of clinical response, which is currently by RECIST criteria, to improved overall survival. We have learned that radiographic progression of disease does not correlate with survival, and may not justify the discontinuation of the treatment, as patients show clinical response regardless of imaging (for example using imatinib in GIST).
2. This concept is becoming more accepted, as we learn more about the heterogeneity of the tumors. Our definition of genomics of the tumor has been revisited after performing diffusional geographic molecular profiling. Examples of this are seen in Non-Small Cell Lung cancer and breast cancers, where the response in PET/CT does not always correlate with prognosis.
3. We monitored surrogate markers, which translate into improved survival. These serum and imaging markers have been suggested to correlate with outcome. This has been shown in our research, as well as by other colleagues, where it has been postulated in the literature, but not defined. Two markers, hepatocyte growth factor and VEGF, have been theorized to distinguish cancer patients from normal individuals, and suggested in our research to be prognostic, along with other markers, which we have defined for the first time.

These markers include circulatory tumor cells, along
We also learned that angiogenesis is related to the presence of circulatory tumor cells (CTCs), and that contrary to the common belief that CTC’s are the result of angiogenesis, we described the dissemination of cells as a cause for new vessels. Therefore, we aimed to treat the cause of dissemination of these cells into the bloodstream, and to reduce or eradicate the circulatory tumor cells by reducing the process of epithelial-mesenchymal transition (EMT).

As we attempted to translate the outcomes to a more sustained response, we were obligated to use epigenetic therapies, to change the fingerprint of the tumor. We learned from many clinical trials that the epigenetic fingerprint defines the tumor response to any other therapy. We also learned that angiogenesis is affected by epigenetic translation, as we saw a clear relationship between the ubiquitination of HIF by VHL in renal cell carcinoma, and the transcriptionally silenced VHL, (seen in 20% of cases with clear cell RCC), fails to degrade the HIF. This produces the same result as when the tumor is stimulated by presence of hypoxia and related hypoxia induced factors. Interestingly, the chemotherapy Topotecan can inhibit HIF-1; however many studies have pointed to HIV-2 as the key element in VEGF production.

We already knew that patient survival is only affected by decreased potential for tumor migration and metastasis. Angiogenesis is the key to such event. What we have discovered is an effective anti-angiogenic therapy that does not trigger the tumor by damaging stroma, but rather enhances the ability of the tumor to functionally behave normally. This is accomplished by changing its genomic expression, through epigenetic impact.

3) We treat a tumor’s cell biology as a whole system, rather than dissecting it into different pathways and targets. In this general approach, we advise that the interaction of cell with the stroma and other microenvironmental factors be included.

4) We use therapy that has minimum negative effect on the patient. In order for any therapy to target the entire cancer cell biology, we must use a combination of several drugs to achieve this. The therapy must also have selectiveness in targeting the genes that are involved in tumor progression and migration. For any treatment to have a substantial general effect, broad enough to cover as many genes as possible, and at the same time, narrow enough to exclude normal genes and onco-suppressors, we would need a very delicate combination of drugs.

Our prior data has clearly shown that any multi-targeted therapy is superior in survival advantage over single target therapies. (such as improved survival in RCC using Sorafenib). Prior examples of drug failure have led us to consider using regimens of drugs that induce minimum resistance. Therefore, we have explored the mechanisms of resistance to each category of drug we selected, and we formulated a sequential multi-targeted therapy that aims to reduce the chance of cross resistance.

Since we have learned from our prior experiments that treatment should have harmony with cancer interaction with the stroma, we must target the genes that are involved in carcinogenesis. The more effectively we target the root of the disease, the better success we would have in eradicating it. We have learned that the root of angiogenesis is hypoxia; therefore instead of targeting VEGF, (which the branch or the effect) we have targeted the cause of hypoxia.
Case Study

57 year old female with history of renal cell carcinoma, diagnosed in 2006 and treated with right nephrectomy. She was in remission until September 2014, when a suspicious mass was found in the contralateral kidney (left side) seen on ultrasound. It was monitored until January 2015, when the imaging (U/S and CT) confirmed its enlargement to 5 cm. This mass was never biopsied, however the chest/abd/pelvis CT in January 2015 showed many other lesions in her lungs, liver, and possibly bone (pelvic), secondary to recurred Stage IV renal cell carcinoma. It showed that there was numerous nodules in both lungs, however she was asymptomatic at that time. She opted for conventional treatment, which was started in March 2015. She was started on Sunitinib at 50 mg per day, and had local radiation to the pelvic bone where the lesion was found. She decided to seek alternative therapies, as she experienced severe side effects from the Sunitinib. She traveled from Canada, where she lived, to see us in California on 5/1/15.

She was experiencing thrombocytopenia (plt of 80), stomatitis, and hypertension. Her initial evaluation revealed extensively high VEGF in her plasma (717) measured on 5/1/15, along with elevated LASA at 22. Her blood was also sent to Biofocus to evaluate for circulatory tumor cells. The test came back positive for c-Myc, G250, and HDAC and DNMT overexpressing CTC (5/13/15). (Please see below.)

Her circulatory tumor DNA reported by Guardant 360, which also revealed positive genomic alterations of VHL and RAF-1, dated 5/11/15.
As part of initial evaluation she was restaged with a whole body PET/CT scan that revealed new lesions in her thyroid, and para-aortic LN. The pelvic lesion was not seen, and other lesions in her kidney and lungs were reported slightly smaller.

She was started on IV daily epigenetic therapies and the dose of Sunitinib was decreased to 37.5 mg a day, on schedule of 4/2 on/off therapy and further stopped. Her stomatitis was resolved, she felt better, blood pressure stabilized and was reduced. Her platelets were rechecked and had normalized.

Her CTC was repeated after the 10 IV epigenetic therapies per MTET (multitargeted epigenetic therapy) protocol, on 5/28/15. The CTC assay showed complete resolution of the circulatory tumor cells that were present in the blood. These cells had demonstrated G250 activity, c-Myc, as well as Histone deacetylation and DNMT activity. All these markers completely disappeared in the second sample from the patient post therapy. (Please see below.) Her VEGF was repeated and showed a reduction from 717 to 303. It was drawn again on 6/12/15 and normalized at 108. Her LASA normalized at 14.

Her ctDNA was retested on 6/12/15 through Guardant 360 laboratory after 4 weeks of treatment. It showed complete resolution of RAF mutation.

She is continuing treatments in our clinic and so far has responded beyond expectations, and surpassed her overall expected survival of 6 months. This excellent response in a short time signifies that this regimen of treatment holds great promise and needs further trials.

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### Report 1 Prior to therapy.

**Analysis Report**

**Patient:** Tumor Patient: Renal CA

For the analysis, we performed the following work steps.

#### 1. Isolation of circulating tumor cells / micrometastases

Circulating tumor cells were isolated from the patient's peripheral blood. A preparation of mononuclear cells (MNC) served as a 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:

<table>
<thead>
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<th>Interpretation</th>
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<td><strong>Telomerase</strong></td>
<td>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. <strong>neg:</strong> Expression of telomerase was not detected in the isolated cells.</td>
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<td><strong>C-MYC</strong></td>
<td>Overexpression of C-MYC indicates an increased proliferation-rate of the isolated cells. <strong>pos:</strong> The expression level of C-MYC was elevated.</td>
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<td><strong>CK19</strong></td>
<td>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. <strong>neg:</strong> There was no expression of CK19 detected.</td>
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<td><strong>G250</strong></td>
<td>G250 is a tumor associated protein which serves as diagnostic marker for renal cell cancer. Thus, the detection of elevated expression of G250 mRNA indicates the presence of circulating renal cancer cells. <strong>pos:</strong> elevated expression of the G250-gen detected.</td>
</tr>
</tbody>
</table>

#### Interpretation

In the isolated tumor cell fraction, elevated expression of C-MYC and G250 was observed.

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### Report 2 Eradication of the ctc's after epigenetic therapy.

**Analysis Report**

**Patient:** Tumor Patient: Renal CA

For the analysis, we performed the following work steps.

#### 1. Isolation of circulating tumor cells / micrometastases

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

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<td><strong>C-MYC</strong></td>
<td>Overexpression of C-MYC indicates an increased proliferation-rate of the isolated cells. An increased proliferation-rate is a typical feature of tumor cells. <strong>neg:</strong> The expression level of C-MYC was not elevated.</td>
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#### 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.
References


Conclusion

We conclude that a “Multi-Targeted Epigenetic Therapy” can maximize the response defined earlier by our surrogate markers for survival. We call this “MTET”, and we have proved our concept in the clinical settings by treating over 100 patients with advanced cancer, and showing clinical efficacy. The clinical advantage was seen in a range from complete remission for over 15 months, to improved markers that correlated with improved survival.

Such novel treatment is merely designed as a result of critical investigation of the magnitude of failures previously described, and clinically tested therapies aimed at inhibiting angiogenesis. We are convinced that the current standard of care will be significantly impacted by introduction of this method, as it statistically manifests itself as a superior approach to treat cancer in a large spectrum of tumors.