siRNA as a Treatment for Melanoma

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By
Luke Vaughn

Thesis Advisor
Dr. Clare Chatot

Signed

Ball State University
Muncie, Indiana

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Abstract

Melanoma is a cancer of the melanocytes (pigment-forming skin cells) and it takes more lives today than ever before; treatment methods simply cannot keep up with the climbing rate of incidence (1). Although melanoma overall has a 5-year survival rate of 91% and represents only 5% of skin cancers, it is still responsible for 2.7 fold more deaths than all other skin cancers (30). Advancements in the understanding of oncogenic signaling, however, have led to a promising new wave of melanoma treatments (3). These treatments center around BRAF, a gene that when mutated, is believed to be a cause of melanoma. BRAF, like many genes is transcribed into a molecule called mRNA, which is then translated into the functional product, a protein. One of these drugs, Vemurafenib, was associated with a 63% reduction in death when compared to decarbazine (a common chemotherapy drug) in a phase III study (5). Vemurafenib gets is name from its function, V600E mutant BRAF inhibition. Unfortunately, as with most BRAF-V600E inhibitors, Vemurafenib’s high initial success rate is followed quickly by resistance to the drug (3). While Vemurafenib acts as a protein kinase inhibitor for the mutated protein product of V600E, the treatment proposed by this paper is to instead target the mRNA of V600E for degradation by siRNA. This treatment will focus on stopping the problem one step earlier in the process than inhibitors such as Vemurafenib. If siRNA is able to knockdown BRAF-V600E, it could be used alongside drugs such as Vemurafenib to improve the current treatment of melanoma.

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Introduction

The “Battle Against Cancer” is a phrase everyone in the modern world is familiar with. It is an ongoing struggle that began with the discovery of cancer by ancient peoples. It has not been until the last century however, that any progress has been made against it. Every major advancement that has been made in treating this disease has come directly from changes in the basic understanding of cancer. The understanding of cancer is expanded through research advancements and then application of those breakthroughs gleaned from research. The focus of this paper will be to use the understanding of a specific form of cancer, V600E induced melanoma, to propose a new treatment for it.

Melanoma is a cancer of the melanocytes (pigment-forming skin cells) and it takes more lives today than ever before; treatment methods simply cannot keep up with the climbing rate of incidence (1). Although melanoma overall has a 5-year survival rate of 91% and represents only 5% of skin cancers, it is still responsible for 2.7 fold more deaths than all other skin cancers (30). The key to the overall high survival rate is early detection, effective staging, and then appropriate treatment based on the staging. Treatment based on staging is important in the balance of minimizing damage to tissue against preventing recurrence of the cancer. Melanoma is staged by the size of the tumor and the degree of spread of the cancer. In stage I the thickness is less than 1.0mm and may be ulcerated. For stage II, the tumor may be up to 4.0mm and ulcerated but not metastasized. Stage III melanomas have spread to the lymph nodes but not other areas of the body. In general, earlier stages of melanoma can be cured through excision alone because the cancer is a solid mass.
and can be removed all at once. As the stages progress, excision must be paired with other cancer treatment therapies such as: radiation, chemotherapy, immunotherapy, and targeted therapies because the cancer has spread deeper into the tissue (2). As with all cancers, serious problems arise when the cancer is not detected early or when initial treatment is not successful. Stage IV melanoma is more difficult to treat than the other stages because it has spread to other places on the body. Since the cancer has already spread, removal of local cancer alone is not enough. This is also a problem in cases of recurrent melanoma. The cancer may not have spread to other areas, but comes back after removal.

Advancements in the understanding of oncogenic signaling, however, have led to a promising new wave of melanoma treatments (3). These treatments center around BRAF, a gene that when mutated, is believed to be a cause of melanoma. BRAF, like many genes is transcribed into a molecule called mRNA, which is then translated into the functional product, a protein. B-Raf, the protein product of the BRAF gene, is involved in a phosphorylation cascade that contributes to controlling the cell cycle. The cascade is a series of proteins starting with EGF and EGFR at the membrane, followed by GBR2, SOS and RAS then RAF, MEK and ERK. This chain relays a message from a signaling protein on the surface of the cell to instruct the cell’s DNA to produce a specific protein. The protein usually promotes growth, such as cell division. Any malfunction in this pathway can lead to cancer. Mutant B-Raf (BRAF-v600E) has been found in 50% of melanomas, more than any other mutation, and has shown a 4% increase in the chance of the cancer recurring (4). BRAF-V600E’s apparent role as a cause for melanoma has prompted the creation of new
drugs designed to target and inhibit BRAF-V600E. BRAF-V600E-targeting inhibitors have already shown the ability to reduce tumors.

One of these drugs, Vemurafenib, was associated with a 63% reduction in death when compared to decarbazine (a common chemotherapy drug) in a phase III study (5). Vemurafenib gets its name from its function, V600E mutant BRAF inhibition. Unfortunately, as with most BRAF-V600E inhibitors, Vemurafenib’s high initial success rate is followed quickly by resistance to the drug (3). The chance of surviving melanoma could be increased if treatment with Vemurafenib was followed by a treatment that was able to stop Vemurafenib-resistant cells from proliferating. To slow the growth of Vemurafenib resistant cells, the combined treatment would need to have a different mode of action. While Vemurafenib acts as a protein kinase inhibitor for the mutated protein product of V600E, the treatment proposed by this paper is to instead target the mRNA of V600E for degradation by siRNA.

Antisense siRNA’s are ~100 base pair ds-RNA molecules that are able to prevent translation of a target mRNA. After the protein, Dicer, separates double-stranded siRNA’s, the guide strand associates with the RNA-induced silencing complex (RISC). Because of the guide strand’s ability to base pair with the target mRNA, the siRNA-RISC complex is able to distinguish between cognate and non-cognate RNA and target the cognate RNA, i.e the one that encodes the protein, for degradation. The objective of this research is to show siRNA can be introduced to a BRAF-V600E expressing cell and knockdown the gene. Since the mutant mRNA will be degraded, mutated proteins will not be transcribed. This treatment will focus on stopping the problem one step earlier in the process than inhibitors such as
Vemurafenib. The justification for this treatment is that it addresses a possible cause of resistance to protein inhibitor treatment. One hypothesis on the development of resistance to BRAF-V600E inhibiting drugs is copy number gains (additional copies of the gene are present in the DNA) of mutant BRAF (6). Resistance occurs when more proteins are produced than can be inhibited by the dose of Vemurafenib. This problem could be overcome if the mRNA was targeted instead of or in addition to the protein product itself.

**Literature Review**

While today there is no “cure” for cancer, for much of history there has been no treatment at all. The first step to treating a malady is diagnosing it. In ancient times, even that was a tall order as next to nothing was known about cancer. Those cultures that were able to identify it would come to claim it was incurable or that the best course of treatment was no treatment at all.

Scrolls from ancient Egypt have been found suggesting the authors were familiar with cancer. They likely knew nothing about the disease but they were able to observe its symptoms. The scrolls depict people with tumors in their breasts and the scrolls detail that this malady has no cure (21 page 40, 30).

The ancient Greeks were familiar with symptoms of cancer as well and had an insight into treating it that the Egyptians may not have had. Cancer received its name from the Greek physician Hippocrates. He named it Cancer or “crab” for the fingerlike projections that can be seen streaking under the skin from tumors (21 page 47). Galen, another Greek physician gave it the name “Onkos” for its
characteristic swelling (21 page 47). Both men believed cancer to be a humoral disease. They believed the body was composed of "humors" (vital liquids), and that cancer arose from a humor, a black bile. This incorrect belief was held for centuries and mislead many physicians. However, some good came out of this. The recommended treatment for cancerous lesions was not to attempt to treat them. This was sound advice since recurrence rate after extracting a tumor is high and at the time, there were no ways to operate without causing the patient extreme pain and distress. Ancient peoples took the first step in defeating cancer, which is to identify it. A problem cannot be solved until it is first identified. The Greeks also made an important evaluation that removal of a tumor alone is not a cure for cancer because it will only come back. The treatment was derived directly from what was understood about the disease and the ability to treat it at the time.

Doctors followed the advice of the Greeks for centuries. They did the best they could for a patient with cancer, which was nothing. It was not until people learned to take a look at the body themselves that the first step could be take to effectively treat cancer. In the 16th century, a leap was made in the understanding of cancer, which resulted directly from advancement in the understanding of the human body. Finally, medicine started to step away from the ideas of humors in the body and take an initiative to describe what was truly there. In 1533, Andreas Vesalius began drawing one of the first accurate and exhaustive depictions of the human body, depicting the venation and nervous systems. He drew only what he was able to observe himself, which did not include any of the humors described by the Greeks (21 page 53). A peer of Vesalius, Mathew Baillie illustrated another account of
human anatomy "The Morbid Anatomy of Some of the Most Important Parts of the Body." His drawings depicted illness in the body, including cancerous tumors, with the same objectiveness that Vesalius had used in his illustrations of the "healthy" body. Baillie also, saw no signs of humors in the body, and importantly for the understanding of cancer saw no black bile around tumors (21 page 53).

The new understanding of anatomy led to further curiosity and exploration of the body, usually carried out on animals or cadavers. John Hunter, Ballie's uncle, studied tumors and developed techniques to remove them and from corpses. He described them as "movable" or not. His idea of "movable" tumors would later become instrumental in the staging of tumors. However, the understanding of Cancer outpaced the ability to treat it. For even with the knowledge of when the removal of a tumor was less likely to result in reoccurrence, these techniques could not be easily performed on conscious patients and the risk of infection was high due to the traditions of poor sanitation in medicine. With the advent of ether for anesthesia and soap for sanitation, surgeons were able to start removing lesions with some success. The ability to treat cancer was again dependent on the knowledge of the disease and our ability to treat based on that knowledge.

The first of the theories to replace the Greek humor theory was the blastema theory. This theory correctly suggested that lesions consisted of rapidly dividing cells and were able to spread by cells traveling to other parts of the body. Three men were responsible for these ideas. In 1838 Johannes Muller suggested lesions were composed of cells and that they arose from budding agents, blastemas (7). While he was wrong about the origin of the lesions the idea that tumors were made of cells
was crucial to furthering our understanding of the disease. Rudolf Virchow put forward the idea that cancer spread from preexisting tumors through some secretion of the tumor in 1863(7). Karl Von Rokitansky was able to modify Virchow’s theory by correctly pointing out that tumors spread through malignant cells (8). The idea that cancer was caused by subdividing cells paved the way for treatment outside of tumor excision.

The 1895 discovery of X-Rays revolutionized medicine. The ability to see into the body from the outside provided unprecedented abilities to diagnose patients. For the treatment of cancer, the discovery of radiation led to a treatment still used today, one that is able to target rapidly dividing cells. (21 page 75) Unfortunately since radiation is able to kill cancer cells by destroying their DNA is it also dangerous to regular cells. Around the same time another treatment arose that took advantage of our new knowledge that cancer comes from rapidly dividing cells. Chemotherapy, which is also used today, is the use of chemicals to stop the growth of cancer cells. The most common strategy is to remove some metabolite a cancer cell would need to grow and divide. The reduction of the metabolite leads to slowed growth of cancer. However, normal cells depend on the same resources as cancer cells, so chemotherapy drugs have severe side effects on normally dividing cells.

These two new treatments were able to go beyond simply excising lesions by taking advantage of new understanding of cancer and applying it through other breakthroughs in biotechnology. These treatments are effective at targeting rapidly diving cells, making them good at stopping the growth of cancer but also good at killing normal cells in our body that are naturally quick-dividing. For example, cells
in the gut, blood cells in the bone marrow, and hair follicle cells. This is why you see the common side effects of hair loss in chemotherapy. The next breakthroughs in cancer treatment will be ones that are even better at stopping cancer cells exclusively. The treatments will depend on knowing just what makes a cancer cell different that a normal cell. Enter virology.

Virology, the study of viruses has produced countless breakthroughs in medicine. Obviously, it has led to treatments of viral diseases, but it has also produced valuable information into the mechanisms of cancer. Virology gives us insight into the true nature of why cancer cells divide rapidly; it is a result of the relationship between cancer causing and cancer suppressing genes.

In 1908, Vilhelm Ellermann and Olaf Bang of Denmark were able to infect chickens with leukemia through injection of a cell-free filtrate. At the time, they did not know leukemia was a cancer. However, it later was important for showing cancer was caused by a non-cellular agent (9). In 1911 scientists knew the paradigm of our understanding of cancer had shifted when cancer was caused in a similar way in chickens though a virus known as the Rous Sarcoma Virus (RSV). The experiment proposed a viral origin for cancer (10). However, it was thought that the discovery may not apply to humans because chickens are a poor human model.

That thought changed though in the 1930's when two experiments were able to show a similar results in rabbits. First, an experiment by Richard Shope and Weston Hurst showed that rabbits could be vaccinated against a papilloma virus. Experimental groups were injected with both infectious and non-infectious solutions intravenously or into the body cavity. The solutions were made from
tissue infected with rabbit papilloma. These rabbits and control rabbits were then exposed to the virus 20 days later. All vaccinated rabbits showed immunity or resistance to the exposure (11). Then a 1935 experiment by the Laboratories of The Rockefeller Institute for Medical Research showed that papillomas caused by the virus Shope used were cancerous (12). They determined this observing rabbits that developed papillomas after being exposed to the virus. At the time the paper was published, over 50 malignant tumors had been found in 8 of the rabbits. A viral origin for cancer had now been shown to apply to mammals. A 1936 experiment by Bittner helped support this by providing data from a mouse tumor virus. Adenoviruses were finally shown to cause cancer in lab conditions in 1962 (14). Adenoviruses are a family of viruses that are classified by being found in human adenoids. In 1962 Trentin was looking for human, cancer-causing viruses by injecting filtrates of known human viruses into hamsters. Hamsters injected with human type 12 adenovirus had a significantly high incidence of malignant tumors. The same year a rhesus monkey virus, SV40 (Simian virus 40) was shown to cause tumor formation (15). Defining a viral origin for cancer was able to directly lead to the prevention of those virally induced cancers. For example the Human Papilloma Virus (HPV) vaccine is able to prevent cancer that can result from HPV infection. However, this was not all virology had to offer for our understanding or treatment of cancer.

Besides providing a possible cause for cancer in the form a virus, virology has also been invaluable in establishing the understanding of the role genes play in cancer. In 1970 Duesberg and Vogt were able to compare two strands of RSV, one
that caused cancer and one that did not, to identify the specific cancer-causing gene (17). They isolated avian sarcoma virus B77 (a virus known to cause cancer in birds) from chicken tumors. They then created experimental groups of the virus by exposing it to UV radiation. The radiation exposure would cause the virus to mutate. By determining which specific gene was required to remain intact for the virus to cause cancer, Duesberg and Vogt were able to identify which gene was the oncogene (cancer causing gene). This experiment set the precedence for discovering oncogenes. In 1976 Stehelin, Varmus, Bishop, and Vogt found genes that caused tumors in birds and were closely related to genes normally present in bird DNA (18). They were able to establish that cancer can be caused by mutations in normal genes; these genes are called proto-oncogenes. The next breakthrough was connecting that proto-oncogenes are often genes connected with regulating cell growth and division (16). Common proto-oncogenes include: RAS, WNT, MYC, ERK, and TRK. These protein products of these oncogenes are all part of signaling pathways that regulate cell growth or division. Mutations in these genes lead to proteins that will not do their job correctly. When these mistakes occur, they lead to cells dividing out of control and cancer develops.

In the 2000’s strides were made in refining the molecular model for how proteins that come from oncogenes promoted uncontrolled growth (16). P53 is one of the most important oncogene genes because a p53 mutation is present in 50% of cancers. Given its great importance, its discovery as an oncogene was crucial. However at the time of the discovery, its full significance was not known (19). In 1989 the P53 gene was found to produce a protein that suppressed tumor formation
This means its normal function is to stop tumor growth and a mutation results in its inability to function this way. The difference between P53 and other oncogenes is that most oncogenes signal the cell to divide, whereas P53 signals the cell to stop diving or to self-destruct. These discoveries led to the understanding of the relationship between tumor causing and tumor suppressing genes. This knowledge is on the current battlefield of the fight against cancer; it being used to develop the treatments of tomorrow. The objective of this paper is to use the understanding of the cause of cancer by oncogenic products to develop the next wave of treatments, specifically a treatment for malignant melanoma.

**Methods**

The objective of this proposed experiment is to show that siRNA can target mutant BRAF-V600E RNA for degradation. Two human cell culture lines will be used in this experiment, one expressing wild type BRAF and one expressing V600E. Three siRNA treatments will be compared. BRAF mRNA targeting siRNA will be the positive control because the literature has demonstrated the ability of siRNA to knockdown this gene. V600E mRNA targeting siRNA will be the experimental group. Knockdown in this group will support siRNA as a viable treatment. An siRNA with the same nucleic acid composition of the experimental group but in a non-cognate or random sequence will be used as the negative control. A lack of knockdown will show that it was ability of the siRNA to bind the target mRNA that led to knockdown. All treatments will consist of predesigned siRNA obtained from ThermoFisher Scientific (22). Five replicates of each treatment will be transfected into both wild
type BRAF and V600E expressing cells by lentiviral HIV vectors containing the siRNA treatments and an indicator sequence.

Knockdown of B-Raf and mutant V600E protein and will be assessed by quantitative, western blots. The SDS page will use RIPA and Laemmli buffers and the gel will be 7.5% given the size of BRAF, 84,437Da (24). Li-Cor imaging software will be used to compare the expression levels of protein. The primary antibody for BRAF will be mouse anti-BRAF antibody purchased from Roche (25) and the secondary antibody will be IRDye 800CW goat anti-mouse IgG and be purchased from Li-Cor (28). The primary antibody for V600E will be a mouse anti-BRAF V600E antibody purchased from Roche (25). The secondary antibody will be IRDye 680RD goat anti-mouse IgG (28). Ideally, V600E mRNA targeting siRNA will show reduced expression of V600E mutant protein but not of normal BRAF. To further support that siRNA is able to target V600E for degradation and that siRNA is a viable treatment, a phosphospecific western blot for ERK will be performed. ERK and phosphorylated ERK will be assessed via quantitative western blots as shown by Charles Adelman et al. (27). Primary antibodies will be rabbit anit-phospho-ERK1/2 (D13.14.4E) and mouse anti-total-ERK1/2 (3A7) respectively and will be purchased from Cell Signaling (27). Secondary antibodies will be IRDye 680RD goat anti-rabbit IgG and IRDye 800CW goat anti-mouse IgG and be purchased from Li-Cor (28). Since ERK is a down stream phosphorylation product of BRAF, V600E protein reduction should result in ERK not being phosphorylated. If ERK is not phosphorylated it will support that the treatment was effective in targeting of normal BRAF or V600E and that the cell will not be signaled to divide out of control.
**Discussion**

These experiments could lead to a variety of potential outcomes. Ideally the positive control, predesigned BRAF siRNA will knockdown BRAF and/or V600E, the negative control random sequence will knockdown neither, and the experimental will knock down V600E but not normal BRAF. Significance would be determined by 95% confidence intervals of data from the quantified western blots. These results would support that siRNA can be used to target mutant BRAF and pave the way for future research and possible treatments based on this method. Future studies could include rPCR to assess and further support that BRAF or V600E RNA has been degraded. Animal trials would be used to determine if delivery of the vector is possible in considering this for clinical trials. If siRNA is shown to knock down mutated BRAF and inhibit phosphorylation of a downstream target, a cell proliferation assay will be used to determine if siRNA affects the rate of proliferation and if cells develop resistance to siRNA treatment as they do to existing BRAF-V600E inhibitors. Further, global inhibition of BRAF potentially presents serious side effects. Therefore, additional studies should be performed to create siRNA variants, which can preferentially bind to mutant BRAF transcripts versus wild-type transcripts. Administration techniques for BRAF cognate siRNA as a medication will also be developed with the objective of delivering the siRNA to cancerous melanoma cells and not non-cancerous, healthy cells. For example, techniques similar to the DPC (Dynamic polyconjuagates) of Rozema et al.(29) could potentially be used as delivery systems for these siRNAs.
Despite a greater understanding of the physiology of cancer, nearly 1,000,000 people are living with melanoma and an estimated 10,000 died from it last year (National Cancer Institute). Drugs that inhibit mutant B-Raf show promise in helping treat melanoma but have a shortcoming in that the cancer quickly becomes resistant to them. If siRNA is able to knockdown BRAF-V600E, it could be used alongside drugs such as Vemurafenib to improve the current treatment of melanoma. This treatment is limited by its ability to only treat cells susceptible to transfection by synthetic oligo-nucleotides. It will not be a cure for cancer, but when combined with other therapies could provide more efficient treatment and serve as a step towards understanding and ultimately beating cancer.
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