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Review Article

miR-196a Silencing of HoxD8: A Mechanism of BRAFi Resistance

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Abstract

Colorectal Cancer (CRC) is the third most common cancer found in the United States. Similar to many cancers, CRC commonly arises from mutations of the genes found along the Mitogen Activated Protein Kinase (MAPK) pathway. Cancer treatments using inhibitors of the mutated proteins along the pathway have become widely used. Despite the overall effectiveness of these treatments, it is always possible that a pathway of resistance may develop, causing the cancer to become unresponsive to treatment. Previous literature has implicated the mutation of HoxD8, a homeobox gene responsible for embryo development and limb segmentation, as a possible pathway of resistance, though the details of this pathway are unknown. This review seeks to propose such a mechanism. It was discovered that miR - 196a, a type of miRNA, is known to silence HoxD8, which in turn causes the increased expression of STK38. This can either activate the rest of the MAPK or stabilize MYC, affecting the cell's morphology and allowing cancerous cells to continuously proliferate.

Keywords: BRAF; Colorectal cancer; HoxD8; MAP kinase; Melanoma, miR·196a, MYC

Abbreviations: Mitogen Activated Protein Kinase-MAPK; Colorectal Cancer-CRC; BRAF Inhibitor-BRAFi; Death-Inducing Signaling Complex-DISC; Fas-Associated Death Domain Protein-FADD-Glycogen Synthase Kinase 3 Beta-GSK3β

Introduction

Colorectal cancer is the third most common cancer (excluding skin cancers) in the United States. The American Cancer Society estimates that in 2019, there will be 101,420 new cases of colon cancer and 44,180 new cases of rectal cancer. It is also the second deadliest cancer with approximately 50,000 deaths every year [1]. Due to increased screening, the incidence and mortality rates of CRC have declined in adults over the age of 50; however, the incidence rate in young adults has increased by 51% over twenty years and the mortality rate has increased by 11% over ten years [2]. Those living a western lifestyle are at a greater risk of developing the cancer through smoking, being overweight, having a diet that is low in calcium and fiber and not exercising. It has been noted that above the age of 35, men are typically at a higher risk for developing the cancer than women. The difference in the rate between the sexes constantly increases with older age. Additionally, African Americans are at a higher risk of developing it than whites and those of other minorities. Considering this, the current recommendation from the American Cancer Society for colorectal screening is that adults begin screening as early as age 45, with a strong recommendation to begin screening at least by the age of 50. Stool tests should be continued annually until the age of 75 with colonoscopies conducted every 10 years. Additionally, CT colonography and flexible sigmoidoscopies should be conducted every 5 years. Screenings may continue until age 85 depending on the life expectancy of the individual [2].

Most cancers are caused by mutations in the MAPK pathway. While mutations commonly occur at the first step of the pathway, KRAS, they may also occur along the downstream steps of the pathway. An example of such mutation sites is RAF, the next protein in the pathway. It is known that one of the isoforms of RAF, BRAF, can have mutations that can cause tumorigenesis. BRAF mutations are prevalent in melanoma and are also applicable in cases of colorectal cancer [3,4]. Treatments of cancers caused by such mutations have become widespread are continuously being investigated.

BRAFi Resistance

The Mitogen Activated Protein Kinase (MAPK) pathway is a series of intra - cellular signaling proteins that span the cytoplasm and regulate cell division [5]. Mutation of these proteins is commonly the cause of the rapid cell division associated with

cancers. To stop cancerous cell proliferation, a prevalent strategy of drug therapy for patients with cancers caused by MAPK mutations is a treatment regimen with an inhibitor of the specific mutated molecule responsible for effecting the cancer (e.g., RAF MEK, ERK, etc.). Unfortunately, this course of treatment is not always effective since the MAPK can develop mechanisms of resistance to the inhibitor, causing cancerous cell proliferation to continue despite treatment. Mechanisms of acquired inhibitor resistance have been the subject of recent investigation. BRAF inhibitor (BRAFi) resistance has been widely investigated in melanoma, the cancer where BRAF mutations are most widely prevalent. Examples of such mechanisms include the dimerization of different isoforms of RAF (ARAF and CRAF), ERK mutations and up-regulation of Platelet Derived Growth Factor Receptor Beta (PDGFR-β), a type of receptor tyrosine kinase, among many others [3,6].

HoxD8 is a Member of the Homeobox Gene Family

It has been previously identified that a mutation of HoxD8, a homeobox transcription factor responsible for limb and anterior/posterior segmentation in embryogenesis, could be a potential cause for BRAFi resistance, though its role in the mechanism of resistance has not been fully identified [3]. Many scientific literatures have thus far implicated mutated homeobox genes in cancer development [7]. Amongst the flour classes of Hox genes (HoxA, HoxB, HoxC and HoxD), mutations in the member genes of HoxA and HoxD have been implicated in cancers of the endodermic organs (breast and colon), while HoxC has generally been present in lung and prostate cancer [7]**.**

In addition to melanoma, the expression of HoxD8, a sub-member of the HoxD homeobox class, has been extensively documented in CRC. Hox genes appear even post - embryogenesis in adult stem cells. In the colon, stem cells aggregate in the colonic crypt, waiting to replace colonic epithelial cells that are damaged due to disease or injury. Hox has a role in differentiating these cells as well [8]. The continued presence of Hox in adult cells can give rise to tumorigenesis, either through the transcription of undesired genes or through the repression of others [8]. Hox genes have been reported to be either up or down regulated in certain cancers. In the case of CRC, it has been established that HoxD8 is down regulated [9] and therefore, could potentially cause drug resistance through the pathway proposed in this study.

Relevant Molecules to the Proposed Mechanism

(STK38, MYC, GSK3β and AXIN1)

Cellular attachment occurs via a variety of connections involving an array of different fiber types. For example, epithelial cells attach to each other using epithelial cadherin (E-cadherin). In an E-cadherin network, the actin filaments of the adjacent cells are connected to vinculin and catenin, which are in turn connected to a calcium dependent complex in the intermembrane space in the zonula adherens. Hox down-regulation has been known to decrease the expression of the related genes, causing cellular detachment [10]. Apoptosis due to cellular detachment is known as anoikis. There are two known pathways that trigger anoikis: the extrinsic pathway (death through the triggering of the cell surface death receptors), or the intrinsic pathway (channel formation in the mitochondria, initiating the assembly of the apoptosome).

In the extrinsic pathway, one of the various receptors found in the Tumor Necrosis Factor (TNF) receptor superfamily is activated by the binding of its corresponding ligand. This causes the formation of the Death-Inducing Signaling Complex (DISC). DISC then associates with an adaptor protein such as Fas-Associated Death Domain Protein (FADD). This in turn activates caspase-8, which upon its cleavage and activation in the cytoplasm, can activate caspase-3, caspase-6, and caspase-7, causing proteolysis and cell death [11].

In the intrinsic pathway, Bim, part of the BH3-only protein activating family, is released into the cell. This causes the pro-apoptotic proteins Bax and Bak to translocate to the outer membrane of the mitochondria. Bim and the protein Bid organize Bax and Bak into oligomers. These oligomers cause the formation of an open channel in the outer membrane of the mitochondria. This permeabilization of the membrane causes the release of cytochrome C from the mitochondria, forming an apoptosome with caspase-9 and Apoptosis Protease Activating Factor (APAF). This assembly activates caspase-3, causing cellular apoptosis [11].

Another well-known death mechanism in the cell is mitophagy: macro autophagy that degrades damaged mitochondria. Mitophagy can occur in a number of different contexts. It can occur spontaneously due to cellular stress. It can also occur as a preprogrammed phenomenon during cell differentiation, reverse differentiation, or as a result of basal mitochondrial maintenance. Two molecular pathways are known for mitophagy: one ubiquitin-dependent and one ubiquitin independent. In the ubiquitin-dependent pathway, often activated by cellular stress, PINK1 is stabilized on the outer membrane of the mitochondria. Parkin is then recruited, causing the ubiquitination of other regions of the membrane. Adaptor proteins (e.g. p62, OPTN, NDP52) then bind to the poly-ubiquitin chains, initiating an autophagosome complex with LC3. In the ubiquitin-independent pathway, mitophagy receptors such as BPIN3, NIX and FUNDC1 bind directly to LC3, ultimately causing mitochondrial fission and destruction [12].

STK38 is a ubiquitous Nuclear Dbf2-Related (NDR) kinase that regulates a variety of other transcription factors leading to cell proliferation [13]. Importantly, STK38 has been known to influence the proliferation of the RAS pathway. One study has suggested that STK38 can cause cellular resistance to anoikis via positive promotion of mitophagy. The effect of STK38 on these

processes can cause cancerous RAS-transformed cells to survive without committing apoptosis [14]. STK38 also causes significant changes to cellular morphology when the expression of Epithelial Cadherin (E-cadherin) is decreased due to decreased HoxD8 expression [15]. A combination of these findings suggests that there may be some co-relation. HoxD8 down regulation may cause a decrease in E-cadherin expression, causing the cells to become spherical and detach from the Extracellular Matrix (ECM). The presence of STK38 may ensure their survival and prevent anoikis, causing tumorigenesis.

On a molecular level, STK38 has been known to negatively or positively regulate proteins of the cellular proliferation pathways. It has also been known to negatively regulate MEKK1/2 [16]. MYC is a proto-oncogene that has been found to be deregulated in 50% of human cancers. MYC is a basic helix loop helix zipper that dimerizes with the protein MAX. The complex, referred to as the E-box, binds directly to consensus DNA with the sequence CACGTG or other similar variants [17]. This dimerization must occur since, on its own, MYC does not have a high affinity to the DNA to which it typically binds. The binding of MAX to the protein MAD instead of MYC causes the antagonization of this pathway. Instead of recruiting chromatin-modifying transcriptional activators and acetyl transferases, as what occurs in MAX/MYC dimerization, a co-repressor containing mSin3, N-Cor and histone deacetylases suppresses gene expression during MAX/MAD dimerization [18]. STK38 is known to be a regulator of MYC expression and mediates MYC ubiquitination and turnover [13].

Although MYC is known to preferentially bind to only a select fraction of highly active gene promoter regions, it can access more of these regions should the cell transform and become cancerous [17]. Tumorigenesis is thought to cause the amplified expression of cell growth by increasing ribosomal DNA expression. This increased expression creates ribosomes that transcribe proteins responsible for cell growth and division. MYC mRNA has a half-life of 30 minutes and MYC proteins themselves are rapidly ubiquitinated in a three-enzyme ubiquitin activation and addition process [19]. The short and unstable half-life of MYC mRNA and proteins ensure that they do not become overexpressed. The MYC protein can be stabilized through its phosphorylation via ERK or other Cycle Dependent Kinases (CDKs) of Serine at position 62 (S62). This sufficiently stabilizes MYC to enable signaling for cell growth and division [19].

Glycogen Synthase Kinase 3-Beta (GSK3β) is a member of the GSK3 family, a serine/threonine protein kinase that is responsible for the activation or deactivation of glycogen synthase, an enzyme responsible for glycogen synthesis. In addition to this role, it has also been implicated in the proteolysis of many proteins, as well as the phosphorylation of a diverse number of signaling molecules. In the study of cancers, GSK3 has been found to be either up or down-regulated depending on the type of cancer. GSK3 has been found to be up-regulated in cancers of the pancreas and ovaries, as well as in leukemia. This is an indication that it promotes tumorigenesis in these cancers. On the other hand, it is down-regulated in cancers of the skin and breasts, indicating that it is a tumor inhibitor in such cancers [20].

AXIN1 is a scaffold protein that coordinates protein complexes such as the Wnt, TGFß, SAPK/JNK and p53 pathways. Additionally, it is known to form complexes with DVL, MEKK, GSK3, APC, β-catenin and itself. It can facilitate c-Myc ubiquitination via interaction with GSK3β, PP2A and Pin1 [21]. Failure of AXIN1 to regulate GSK3β-mediated degradation of MYC can cause the indefinite stabilization of MYC, causing indefinite ribosomal DNA expression and ribosomal assembly [22].

Mansour et al. has asserted that HoxD8 is a negative regulator of STK38 and therefore, an increase in HoxD8 expression leads to decreased expression of STK38 and MYC. In cancers where HoxD8 is down-regulated, such as CRC, STK38 and MYC would be up-regulated and therefore stabilized [15].

MiR-196a Inhibits HoxD8

MicroRNAs (miRNA) are segments of RNA that regulate the transcription of genes that are responsible for cellular mechanisms. They are typically transcribed by RNA polymerase II and processed to pre-miRNA by the Drosha and DGCR8 endonucleases. They are then exported to the cytoplasm via exportin-5 where they are cleaved into double stranded mature miRNA by the Dicer ribonuclease. The miRNA binding to Argonaute proteins forms the RNA-Induced Silencing Complex (RISC) [23]. The newly mature miRNA can silence the sequences of other pre-mRNA in the post-transcriptional modification phase [24]. Changes in the expression of miRNA often cause tumorigenesis. This often occurs through genetic alterations, Single Nucleotide Polymorphisms (SNP), epigenetic silencing or defects in miRNA biogenesis [23].

The role of miRNA in silencing critical regulation genes has been investigated in many cancers as a cause of tumorigenesis. Because of this, miRNAs are also being investigated as potential cancer treatments by targeting and silencing genes responsible for the original tumorigenesis [24]. A prominent miRNA in CRC is miR-196a. The sequence of miR-196a is complementary to that of HoxD8, making HoxD8 a potential target for miRNA-mediated gene silencing [25]. In light of this, miR- 196a can be posited as a cause of tumorigenesis and perhaps a potential mechanism through which inhibitor resistance can develop. Furthermore, miR-196a has also been thought to activate MEK, which could resume the proliferation just downstream of RAF in the RAS pathway [25,26]. It is very possible that an increased expression of miR-196a can cause increased cellular proliferation in the RAS pathway despite treatments to inhibit it.

It is noteworthy, however, that while it has been reported that miR-196a has been shown to activate MEK, the Journal of International Biochemistry and Cell Biology has reported that no subsequent phosphorylation downstream to RAF was affected by varying levels of HoxD8 expression [15]. To reconcile this difference, it should be noted that there has been literature to suggest that STK38 may suppress levels of MEK1/2 expression in the KRAS pathway [16]. It is possible that even if MEK is in fact negatively regulated as a result of STK38 expression, MYC stabilization may be a feasible alternative pathway of cell proliferation.

The Proposed Mechanism

Considering the above, it is possible to suggest that there could be an identifiable mechanism for HoxD8-mediated BRAFi resistance. HoxD8 is down regulated in CRC, while up regulated in normal cell lines. It is possible that this silencing can be caused by miR-196a or other miRNAs [25]. BRAFi resistance may originate with increased expression of miR-196a, down-regulating HoxD8 expression and causing an increase in the expression of STK38. This phenomenon can cause an up-regulation of MEK on account of the increased miR-196a expression. Alternatively, the increase in STK38 expression can cause the stabilization of MYC, changing the cellular morphology to favor proliferation and tumorigenesis. A visual representation for this pathway can be found in Figure 1. This article seeks to critically review the potential mechanisms of BRAFi resistance as reported in the melanoma treatment resistance studies, though are equally applicable in CRC treatment resistance. Exploration of this mechanism can prevent the formation of treatment resistance in both cancer types. It is possible that while miR-196a up-regulation traditionally causes an increased expression of MEK, creating a resistance pathway through resumed expression of the MAPK, the up-regulation of STK38 may negate that possibility. This may shift the resistance mechanism to occur instead through MYC proliferation and cell morphology changes.

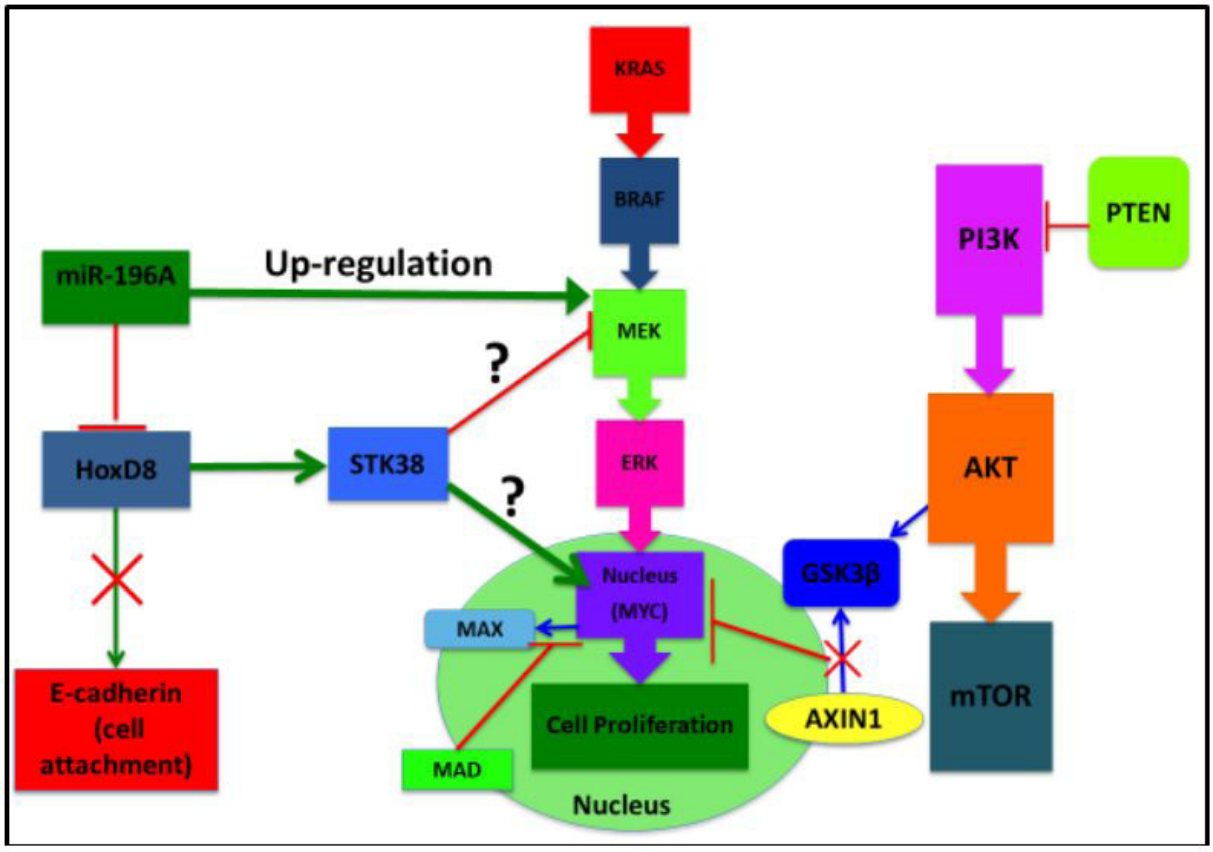

Figure 1: HoxD8 is down-regulated in CRC as a result of silencing via miR-196A. This silencing can cause the up-regulation of STK38 expression, which stabilizes MYC mediated proliferation. Alternatively, miR-196a can directly cause increased expression of MEK. This possibility of cell proliferation may be negated due to the presence of up-regulated STK38, favoring MYC mediated proliferation. A morphological manifestation of HoxD8 silencing is a down-regulation of E-cadherin expression, causing cellular detachment in epithelial tissues. STK38's prevention of anoikis could allow cancerous cells to proliferate into CRC tumors.

Conclusion

Colorectal cancer is a pervasive and deadly cancer found in the United States. Like other cancers, CRC is thought to be caused by mutations in the MAPK and may become resistant to treatment should resistance mechanisms develop before or during treatment. Previous literature has suggested that HoxD8 might be responsible for one of these pathways. We propose that miR-196a may silence HoxD8 expression and cause an up-regulation of MEK, which can resume the expression of the MAPK. Alternatively, via increased STK38 expression, MYC may become stabilized, causing changes to cellular morphology and allowing for tumor proliferation. Further investigation of this pathway may prove useful in discovering and preventing mechanisms of inhibitor resistance. This will provide a greater efficacy of cancer treatment and improved patient prognosis and survival.

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