Characterization of BRAF (V600E) Splice Variants in Metastatic and Drug-Resistant Melanoma

by
Erika M. Sawka

A PROJECT

submitted to

Oregon State University

University Honors College

in partial fulfillment of
the requirements for the
degree of

Honors Baccalaureate of Science in Nutrition
(Honors Scholar)

Presented May 28, 2015
Commencement June 2015

AN ABSTRACT OF THE THESIS OF

Erika M. Sawka for the degree of Honors Baccalaureate of Science in Nutrition presented on May 28, 2015. Title: Characterization of BRAF (V600E) Splice Variants in Metastatic and Drug-Resistant Melanoma.

Abstract approved:

_____

Arup Indra

The BRAF proto-oncogene is a member of the Raf protein family that encodes serine/threonine protein kinases, which activate the MEK/ERK signaling transduction pathway. The V600E mutation of BRAF, found in 70% of melanoma cases, causes an upregulation of MEK/ERK signaling which leads to many hallmarks of cancer, including apoptosis evasion, proliferation, angiogenesis, and metastasis. Protein isoforms resulting from alternative splicing found in BRAF V600E may influence its basal kinase activity in melanocytes and also contribute to drug resistance of melanoma tumors through Ras-independent enhanced dimerization. Two novel splice variants were identified and their relative expression was measured in human samples of non-cancerous melanocytes, BRAF V600E melanoma, and drug-resistant BRAF V600E melanoma. It was discovered that both the abberantly spliced BRAF isoforms that included a 3-nucleotide insert between exon 4 and 5 and another isoform that excluded exon 14 and 15 had increased expression in BRAF V600E melanoma and drug-resistant melanoma. This suggests that

alternative splicing may be a regulatory mechanism that contributes to the oncogenic characteristics of BRAF V600E melanoma and drug-resistance.

Key Words: BRAF V600E, melanoma, MEK/ERK signaling, alternative slicing

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I understand that my project will become part of the permanent collection of Oregon State University, University Honors College. My signature below authorizes release of my project to any reader upon request.

Erika M. Sawka, Author

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Many people have been invaluable to me in completing my undergraduate honors thesis. I began knowing little about performing laboratory work and I have grown to be proficient in many techniques and studies of the skin. Throughout my steep learning curve, countless questions, and success in writing this thesis, I have had a great group of people to support me.

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CHARACTERIZATION OF BRAF (V600E) SPLICE VARIANTS IN METASTATIC AND DRUG-RESISTANT MELANOMA

1. INTRODUCTION

1.1 Skin Structure and Function

Skin, as part of the integumentary system, is the largest organ of the body and functions to provide a protective barrier against pathogens and external environmental damage. Also, the skin has sensory nerve endings, a water-resistant barrier, and sweat glands and blood vessels to perform thermoregulation. The skin is comprised of three main layers of ectodermal tissue, which include the epidermis, dermis, and hypodermis.[1]

The epidermis is the outermost layer and provides the protective barrier of the body (Fig. 1). The layer is mainly comprised of keratinocytes and in addition to these, melanocytes, Merkel cells, and Langerhans cells are also present. The epidermis can be divided into strata from the outermost to innermost: stratum corneum, stratum lucidum (only in palms and soles), stratum granulosum, stratum spinosum, and stratum basale. In the stratum basale, or basal layer, keratinocytes proliferate, differentiate, and become enucleated as they move up through the epidermal layers. Melanocytes populate the basal layer as well and produce melanin, a pigment found in skin, hair, and eyes. The production of melanin, or melanogenesis, provides pigmentation as well as protection of the hypodermis from UV-B radiation.[1]

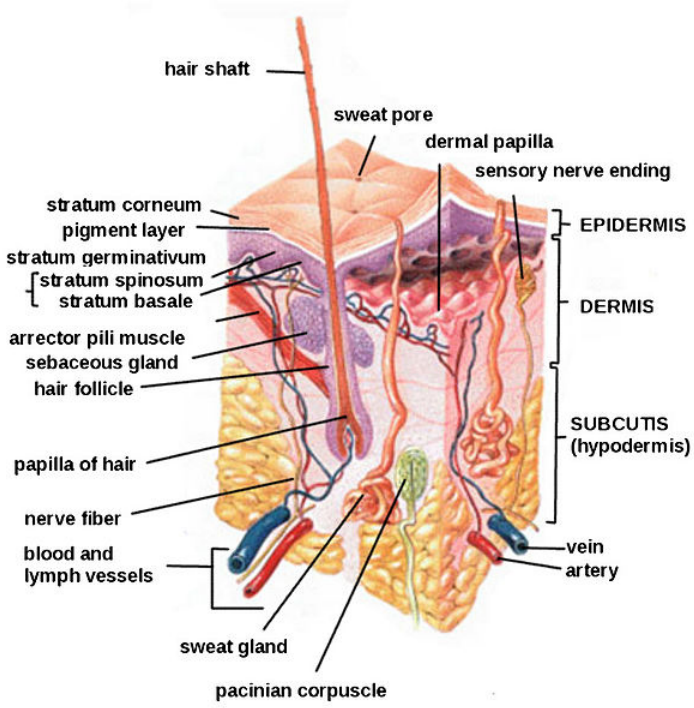

Figure 1: Schematic diagram of the epidermis

1.2 Melanoma Skin Cancer

Melanoma is the most dangerous type of skin cancer and is the leading cause of death from skin disease. Rates of melanoma in the United States have been steadily increasing 1.8 percent each year in the last decade.[2] In 2015, it is expected that 74,000 people will be diagnosed with Stage I-IV melanoma resulting in 10,000 deaths in the United States.[3]

Melanoma is a cancer first characterized by uncontrolled growth of melanocytes during a stage called radial growth phase (Fig. 2).[4] If detected in a patient, this early-stage melanoma can be treated with surgery by removing the tumor completely.[5] However, if left untreated, the proliferating cells will move vertically into the epidermis or lower into the dermis during a stage called vertical growth phase.[4] Once the tumor is deemed

invasive, the tumor has proliferated deeper into the dermis and has the potential to metastasize, in other words spread to the lymph nodes and other sites of the body. The stage of melanoma progression is determined by thickness, depth of penetration, and spread to other tissues. Prognosis of early-stage melanoma is very good, however there is a lack of successful treatments for patients with late-stage, metastasized melanoma.[5]

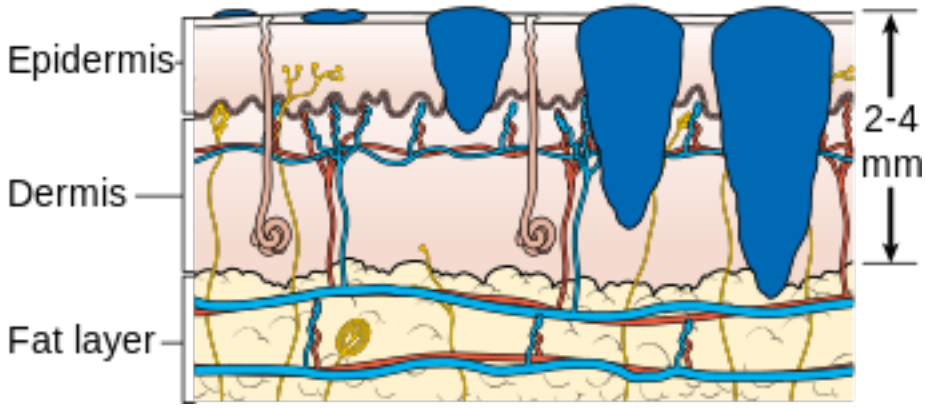

Figure 2: Schematic progression of melanoma

The causes of melanoma are a combination of genetic and environmental risk factors.[3] Family history, attributed to hereditary genes that increase an individual's susceptibility, occurs in 10% of melanoma cases. An individual with fair skin and light hair color have a higher risk of melanoma because less melanin is produced in the skin to protect from ultraviolet radiation. For example, those with the gene for the melanocortin 1 receptor (MCR1), which causes red hair pigmentation, have a higher risk. Environmental risk factors, such as exposure to ultraviolet light from the sun or tanning beds, cause DNA damage in about 90% of melanoma cases. Specifically, cyclobutane pyrimidine dimers, which are common photoproducts of UV radiation, become

mutagenic when unrepaired (Fig. 3).[6] Other risk factors of melanoma include sunburns, a high number of moles, weakened immune system, and age.

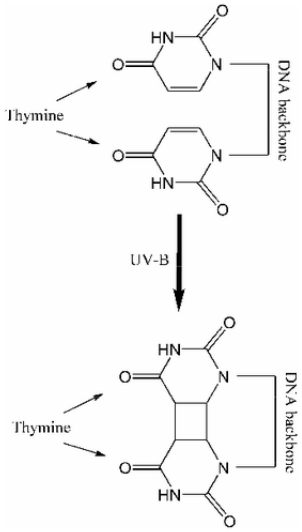

Figure 3: Thymine-thymine dimer is an example of a cyclobutane pyrimidine dimer found in the skin

1.3 Ras-Raf-MEK-ERK signal transduction pathway

Cancer is characterized by an abnormal proliferation of cells, and in the case of melanoma, melanocytes grow out of control. Cells are triggered to begin cell division, or mitosis, when an extracellular ligand binds to a signal receptor on the cell membrane. This induces a signal transduction pathway within the cell that communicates signals via kinase activity, which is the addition of phosphorylation groups from one protein to another in a sequential manner. The final protein of the pathway regulates transcription factors that bind DNA and affect gene expression. This can lead to variability in protein expression that may result in cell fate, such as cell division. In many cancers, there is an oncogenic mutation in one of the proteins that keeps a pathway in an activated or inactivated state to induce constitutive cell division.

The Ras-Raf-MEK-ERK signal transduction pathway plays a role in melanoma tumor survival by providing angiogenic support, evasion of apoptosis, cell survival, migration, and metastasis.[4] The pathway is believed to be upregulated because of oncogenic mutations of the Raf protein family that have been identified in a significant percentage of cancers. Raf proteins are serine/threonine kinases that are activated in a Ras-dependent manner. Raf proteins in turn phosphorylate MEK1/2, which phosphorylates ERK1/2 (Fig. 4). ERK regulates transcription factors that control DNA, which affects protein expression and cell fate, either inducing cell division or apoptosis. The deregulation of ERK causes uncontrolled cell division, leading to the oncogenic properties of a tumor.[4]

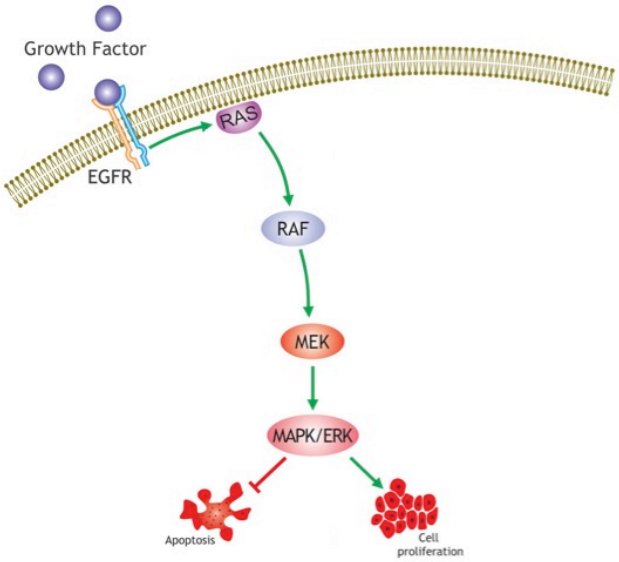

Figure 2: Ras/Raf/MEK/ERK signal transduction pathway

1.4 BRAF mutation and MEK/ERK signaling

The Raf protein family consists of Raf-1, A-Raf, and BRAF and they are activators of the MEK/ERK pathway. Raf-1 is expressed in most tissue types, A-Raf primarily in the urogenital system, and BRAF highly expressed in neuronal tissue. Even though BRAF may be expressed only at low levels in melanocytes, it has been shown that the protein can still have significant effects on MEK/ERK kinase activity. For example, Raf-1 and A-Raf were found to be expressed much higher than BRAF in embryonic fibroblasts in mice, but BRAF had much greater biochemical activity.[4] Studies have also shown that Raf-1 is dispensable in activating the MEK/ERK pathway and rather BRAF is the key activator and will be upregulated in the absence of Raf-1.[4] BRAF has also been shown to induce a higher kinase activity than Raf-1. The BRAF V600E mutation specifically accounts for a 3-fold hyperactivation of kinase activity due to its introduction of negative charges that mimics constitutive phosphorylation.[7] Thus, while expression of BRAF and its isoforms in melanocytes are unclear, it is known that BRAF is a significant MEK/ERK activator in these cells.

The *Braf* gene is a frequent target of mutation due to mismatch repair deficiencies because of its resulting oncogenic activity.[4] A sequencing screen of human cancer samples led to the discovery of BRAF mutations existing in 70% of malignant melanoma and 15% of colorectal cancer.[4] The majority of these BRAF mutations were found to have a substitution of valine with glutamate at codon 600 (V600E) (Fig. 5). Thus, BRAF V600E was found to be the most common mutation of BRAF and was present in over 50% of melanoma cases.[8] The substitution of glutamate in this mutation introduces a negative charge in the kinase domain of conserved region 3 and mimics the negative

charge of phosphorylation.[7] This bypasses the need for extracellular ligands and subsequent kinase signaling. This is thought to contribute to the high basal kinase activity of BRAF, which is 15-20% higher than Raf-1.[4] Thus, BRAF V600E causes a constitutive activation of the MEK/ERK pathway.

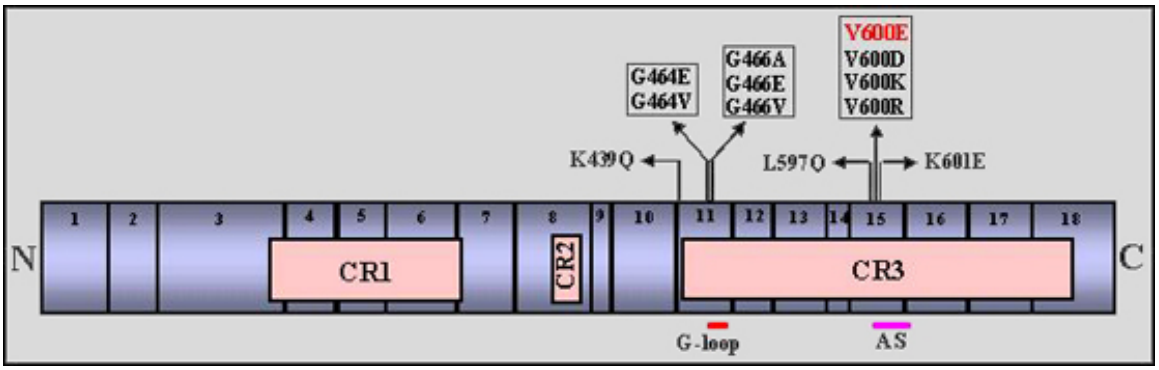

Figure 3: V600E Mutation in BRAF[10]

BRAF has not been found to be the cause of tumor emergence, rather a prerequisite for tumor development.[4] A previous study concluded activating BRAF mutations occur in premalignant nevi and can curiously remain senescent for many years.[9] Other genetic mutations, such as the *INK4* gene perhaps activated by UV radiation, seem to be necessary in order for the cancer to progress. In combination, *INK4* and BRAF V600E induces constitutive MEK/ERK activation and hyperproliferation of benign tissue.[4]

The downstream results of this constitutive activation are increased transcription of a number of proteins. Increased transcription of cell cycle proteins such as cyclins D and E occurs.[4] Other studies have noted higher concentrations of cyclins D and E in dysplastic nevi and metastatic melanoma and they play a role in hyperproliferation by frequent transition from the G1 to the S phase.[11] MEK/ERK deregulation may also induce

mutations in the p53 gene, which influences apoptosis evasion, limitless proliferation, and angiogenic support.[12] ERK deregulation increases the expression of integrins that result in cell migration and metastasis.[13] In summary, BRAF mutations, through upregulation of MEK/ERK activity, promotes the characteristics of tumor progression (Fig. 6).[4]

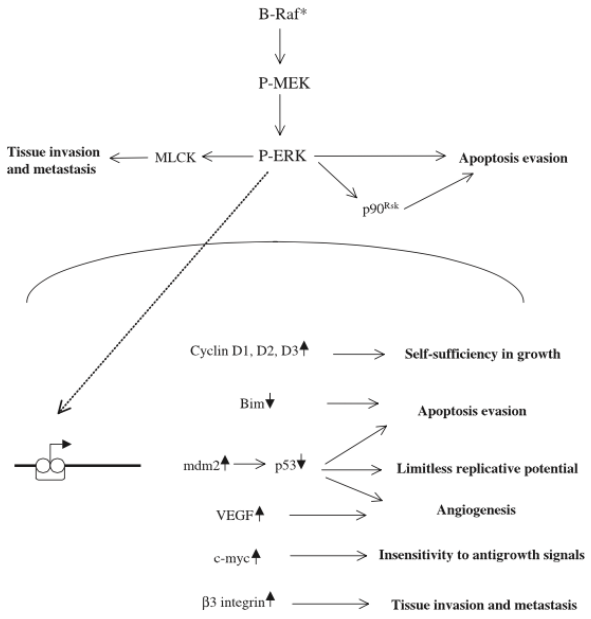

Figure 6: Contribution of activated BRAF to tumor progression and malignancy[4]

1.5 Alternative splicing of BRAF V600E

Alternative mRNA splicing is a post-transcription mechanism that removes the non-coding introns and variably joins the remaining exons. Alternative splicing occurs in about 60-70% of human genes.[14] This mechanism enables the generation of different mRNA isoforms from a single gene comprised of many exons, such as BRAF. These different isoforms, or splice variants, each exhibit tissue-specific patterns of expression and allow a large diversity of proteins with potential differences in structure and

function.[5] Some protein splice variants that result from this alternative splicing may influence the basal kinase activity of BRAF in melanocytes and activate the signal transduction pathway more frequently through constitutive phosphorylation.[4] These splice variants of interest could be the cause of uncontrolled cell proliferation. Thus, alternative splicing is a regulatory mechanism that could influence the progression of malignant melanoma.

2. RESEARCH FOCUS

2.1 Discovery of novel splice variants

Currently it is unknown which of these splice variants are preferentially expressed in melanocytes and malignant melanoma.[4] The BRAF splice variants need to be investigated for their pattern of expression and their ability to activate the MEK/ERK signaling pathway. This would indicate that certain splice variants contribute to the tumor's oncogenic properties and could be targeted for future therapy. There are many reported splice variants of BRAF that have been shown to have effects on BRAF's basal kinase activity.[7,15] Overall, previous studies have supported that the naturally-occurring isoforms of BRAF in mice and humans can modulate its affinity for MEK and induce its kinase activity, thus contributing to oncogenic properties of melanoma.[7]

Dr. Arup Indra's lab discovered two novel splice variants present in BRAF V600E mouse melanoma cells that have not been previously reported. A member of Dr. Indra's lab, Dr. Daniel Coleman, isolated the spliced RNA from mouse melanoma cells that had the BRAF V600E mutation and reverse transcribed it to cDNA. PCR was used to amplify the coding region of *Braf* and the PCR products were inserted into plasmids. The plasmids were sequenced and the cloned splice variants of the *Braf* V600E gene were then aligned to the wild-type *Braf* genes using ClustalW. The novel splice variants that had not been previously reported were identified (Fig. 7).

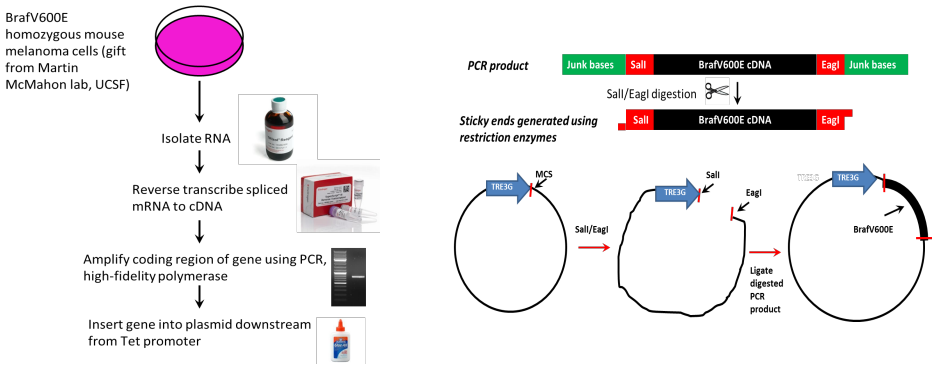

Figure 7: Cloning scheme for identification of splice variants in mouse BRAF V600E melanoma

One of the discovered splice variants of BRAF V600E was a 3-nucleotide insert between exon 4 and 5 located in the Ras-binding domain located in conserved region 1 (Fig. 8). For this paper, these splice variants will be labeled exon 4/5 and exon 4/5 with insert. The second newly identified splice variant was a deletion of exon 14 and 15 the kinase domain located in conserved region 3 (Fig. 9). These splice variants will be labeled exon 15/16 and exon 13/16.

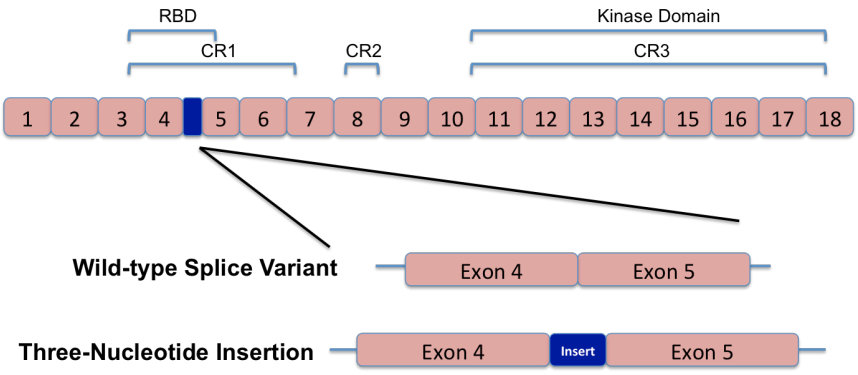

Figure 8: BRAF V600E splice variants exon 4/5 and exon 4/5 with insert

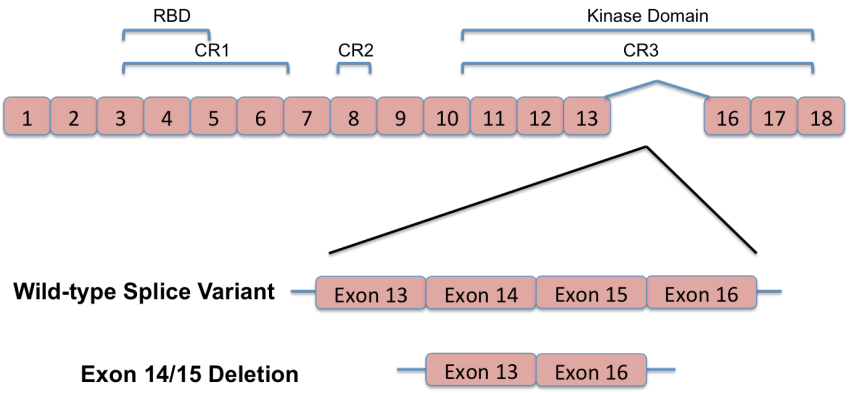

Figure 9: BRAF V600E splice variants exon 15/16 and exon 13/16

Both mouse and human wild-type melanocytes and BRAF V600E-mutated melanomas will be tested for expression of these splice variants. An increased expression of a splice variant in BRAF V600E melanoma, the most common BRAF mutation, may indicate a role in tumor development.

2.2 Drug resistance in BRAF V600E melanoma

Alternative splicing may also be a regulatory mechanism that induces drug resistance. Vemurafenib is a drug that directly inhibits BRAF V600E and interrupts the MEK/ERK signaling pathway (Fig. 10). It's commonly used in melanoma cases with this BRAF V600E mutation, but in the majority of cases, resistance to the drug occurs.[16] Despite frequent resistance to Vemurafenib, this drug has been successful in tumor regression and improved survival, another indicator that BRAF is an important activator of this pathway and malignant melanoma.

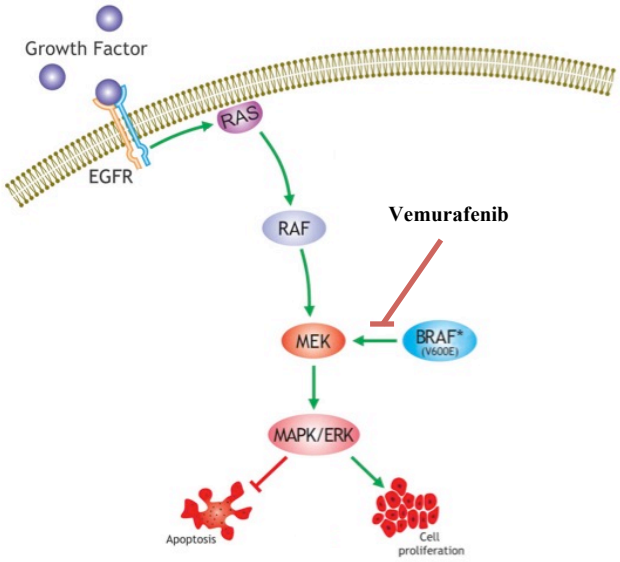

Figure 10: Mechanism of Vemurafenib, a BRAF V600E inhibitor

There are many mechanisms through which the tumor may potentially acquire drug resistance.[17] A few examples include a Ras mutation that bypasses the BRAF step, a MEK mutation, and overexpression of COT (MEK kinase kinase). Another possibility is the over-expression of specific BRAF splice variants which may cause BRAF dimerization independent of Ras. This would increase MEK/ERK signaling to overcome sensitivity to Vemurafenib. Alternative splicing will be the mechanism that is focused on in this project.

A previous study identified a number of splice variants that were expressed in Vemurafenib-resistant BRAF V600E melanoma (Fig. 11).[18] The splice variants discovered in Dr. Indra's lab have never been previously identified in malignant melanoma, including this study, supporting the novelty of these newly found isoforms. If we analyze the expression and activity of these BRAF splice variants in BRAF V600E drug-resistant cells, we could identify a new target for melanoma treatment.

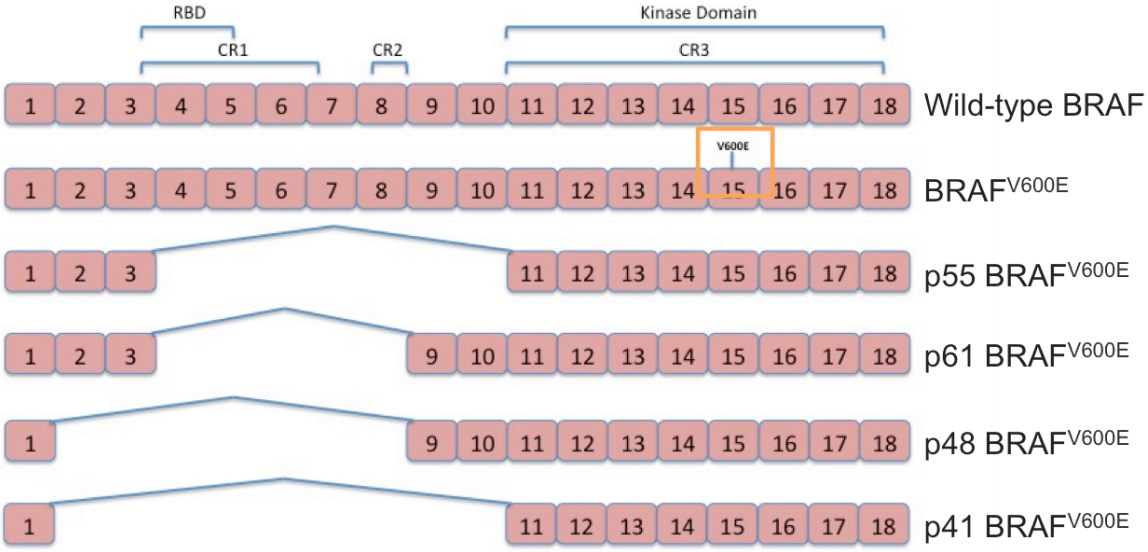

Figure 11: Previously reported splice variants expressed in drug-resistant BRAF V600E melanoma

3. MATERIALS AND METHODS

3.1 Mouse and human samples

Mouse melanocytes were purchased from ATCC and the BRAF V600E melanoma cells were given as a gift from Dr. Martin McMahon's lab at the University of California, San Francisco. Human melanocytes from Donors 1, 2, and 4 were given as a gift from Dr. Pam Cassidy from the Department of Dermatology at Oregon Health & Science University (OHSU). Human melanocytes of Donor 3 were given as a gift from Dr. Zalfa Abdel-Malek at University of Cincinnati. The human samples of BRAF V600E melanoma cells (A375 and WM115) and the drug-resistant BRAF V600E melanoma cells (A2058) were purchased from ATCC.

3.2 RNA extraction and cDNA synthesis

All of the cell samples received were cultured in high glucose DMEM supplied with 10% FBS and penicillin/streptomycin. The drug-resistant cells (A2058) were confirmed to be drug-resistant through additional treatment of Vemurafenib. An initial 5-micromole concentration of Vemurafenib was administered and after 24 hours and substitution of fresh medium, a 10-micromole concentration of Vemurafenib was added After 24 hours and substitution of fresh medium, a 30-micromole concentration of Vemurafenib was again administered for 48 hours which resulted in only 10% of the cells surviving. Fresh medium was supplemented for 24 hours after which the morphology indicated that they were healthy, resistant cells. Thus, two distinctive samples, though both initially drug-resistant, were made by treatment with, or absent of, additional

Vemurafenib. RNA was extracted from the cultivated cells and reverse transcribed to cDNA with the Invitrogen Superscript III kit.

3.3 Real-time qPCR

Real-time quantitative PCR (RT-qPCR) is the most sensitive method available to determine gene expression levels.[14] RT-qPCR can be applied to the detection of splice variants based on simultaneous amplification of different cDNA templates with primers specific to the alternatively-spliced region. The SYBR Green, a DNA-binding dye, provides the fluorescent signals that allow the accumulation of amplified transcript to be viewed every cycle in "real time." Expression levels are then measured by relative quantification; in other words, the expression of the mRNA target related to an endogenous reference transcript. The cDNA acquired from the samples were equalized in concentration and combined with master mix and the primer sets. The endogenous controls, or housekeeping genes, included in the master mix was hypoxanthine-guanine phosphoribosyltransferase (HPRT) for the mouse samples and large ribosomal protein (RPLPO) for the human samples. These controls normalize the measured expression and allow for relative expression quantification.

RT-qPCR was performed in an Applied Biosystems 7500 System using the BRAF primers (Table 1). Quantification was done by fluorescence analysis of SYBR Green. Neglecting the abundance of other isoforms, the amount of alternative splicing was calculated using the comparative C_T method. The C_T value is indicative of the cycle number at which the target signal crosses the threshold value of fluorescence and is essentially a measure of the expression of the target sequence, in this case the splice

variant.[19] The C_T values provided by the Applied Biosystems program were converted to RQ values (relative expression) in Microsoft Excel through arithmetic calculations of the comparative C_T method. The results were standardized relative to a calibrator, or a reference value, in order to reveal comparative expression. The reference value was generally chosen to be the expression of the wild-type splice variant in the melanocyte sample. Overall, the resulting RQ values represent relative expression that is normalized by an endogenous reference gene and comparative to the chosen calibrator.

Sample	Splice Variant	Direction	Name of Primer
Mouse	Exon 4/5	Forward (F)	MH_BRAFSV45X
		Reverse (R)	M_BRAFSV5R
	Exon 4/5 with insert	F	MH_BRAFSV45I
		R	M_BRAFSV5R
	Exon 15/16	F	M_BRAF15072214
		R	M_BRAF16R
	Exon 13/16	F	M_BRAF1316
		R	M_BRAF16R
Human	Exon 4/5	F	MH_BRAFSV45X
		R	H_BRAFSV5R
	Exon 4/5 with insert	F	MH_BRAFSV45I
		R	H_BRAFSV5R
	Exon 15/16	F	H_BRAF15
		R	H_BRAF16R
	Exon 13/16	F	H_BRAF1316
		R	H_BRAF16R

Table 1: RT-qPCR primers to detect BRAF splice variants

3.4 Statistical analysis

These standardized RQ results were then graphed and analyzed using Graph Pad Prism software and unpaired t test. The results utilize stars to indicate the significance of the fold differences between the relative expression of splice variants (Table 2).[20] The

table below illustrates the statistical significance for the varying amount of stars seen on the graphs in the following results section.

Star(s)	Statistical Significance	P value
ns	Not significant	≥ 0.05
*	Significant	0.01 to 0.05
**	Very significant	0.001 to 0.01
***	Extremely significant	0.0001 to 0.001
****	Extremely significant	< 0.0001

Table 2: Statistical significance and P values represented by stars

4. RESULTS

4.1 Analysis of exon 4/5 splice variant expression in mouse melanocytes and mouse BRAF V600E melanoma cells.

Following RT-qPCR, the splice variants of interest were characterized based on relative expression to the calibrator (reference gene). The calibrator chosen for statistical analysis was BRAF splice variant, exon 4/5, in the melanocyte sample. This sample represents a benign melanocyte in a healthy patient with wild-type splicing in the *Braf* gene. The other expression values indicate a fold-change relative to the calibrator value set at 1.0. A significant 0.55-fold decrease in relative expression of exon 4/5 was observed in BRAF V600E melanoma cells compared to melanocytes (P = 0.0093) (Fig. 12). Exon 4/5 with insert had a 0.36 fold higher expression compared to exon 4/5 in BRAF V600E melanoma cells (P = 0.0194).

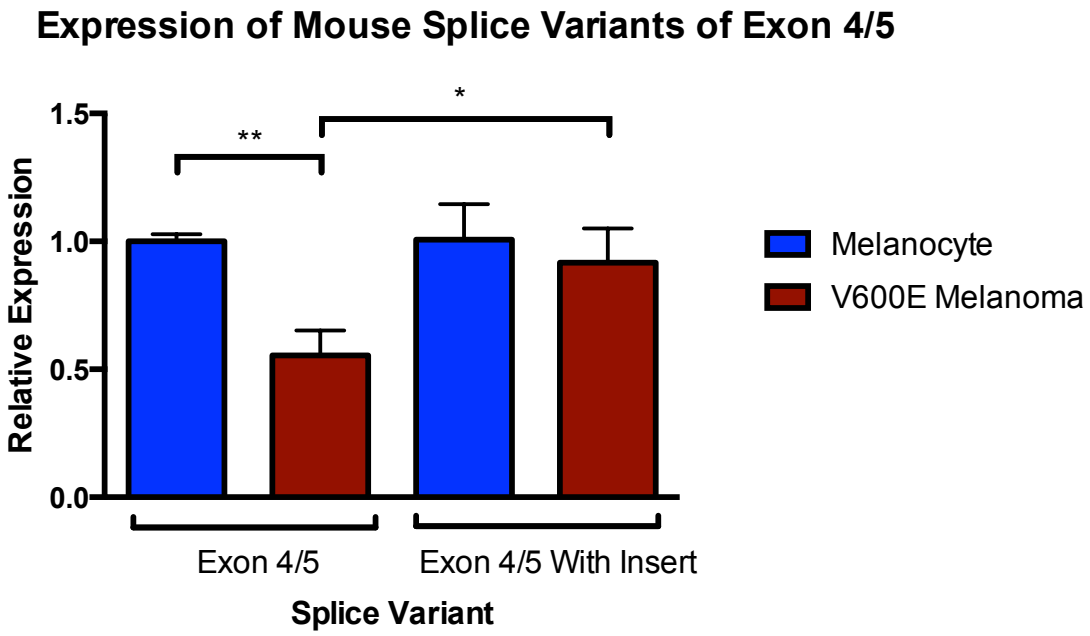

Figure 12: *RT-qPCR analysis of splice variants on multiple mouse samples. The relative expression of the reference isoform (exon 4/5) and the variant isoform (exon 4/5 with insert) was analyzed in melanocytes and BRAF V600E melanoma. The error bar indicates standard error of mean (SEM) of triplicate qPCR reactions.*

4.2 Analysis of exon 4/5 splice variant expression in human melanocytes and human BRAF V600E melanoma cells.

For the human samples, a new calibrator was chosen to represent a benign melanocyte with wild-type BRAF splicing. Any of the four donors of the non-cancerous melanocyte samples would have been acceptable calibrators; however, there was undetected expression of exon 4/5 with insert in Donor 1, 2, and 3. Thus, Donor 4 for exon 4/5 was chosen to be the calibrator and the other values represent a fold-change relative to the calibration value of 1.0. A significant increase in expression of exon 4/5 was observed in both BRAF V600E melanoma cell lines (6.52 fold in A375 and 3.46 fold in WM115) compared to the reference melanocytes ($P = 0.0003$; $P = 0.0059$, respectively) (Fig. 13). Similarly, a significant increase in expression of exon 4/5 with insert was observed in both BRAF V600E melanoma cell lines (133.83 fold in A375A and 66.41 fold in WM115) compared to the reference melanocytes ($P = 0.0004$; $P < 0.0001$, respectively). When analyzing only BRAF V600E melanoma cells (A375 and WM115), expression of exon 4/5 with insert was markedly higher by 127.3 fold and 62.95 fold, respectively, compared to exon 4/5 ($P < 0.0001$; $P < 0.0001$, respectively).

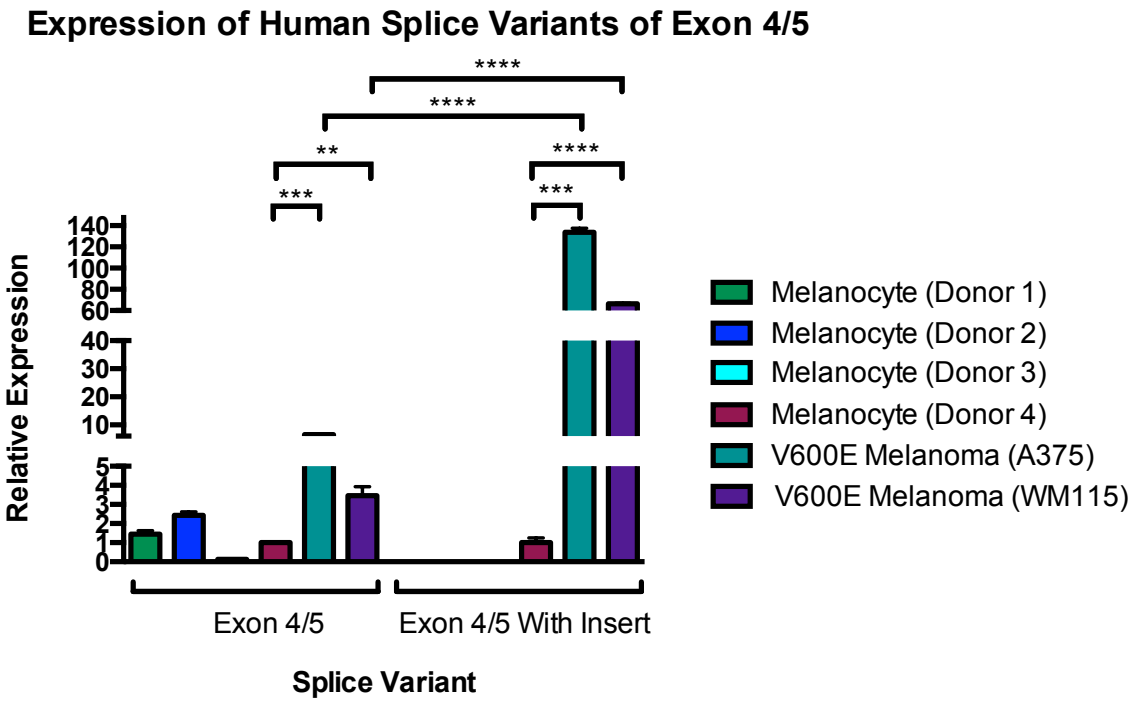

Figure 13: *RT-qPCR analysis of splice variants on multiple human samples. The relative expression of the reference isoform (exon 4/5) and the variant isoform (exon 4/5 with insert) was analyzed in melanocytes and BRAF V600E melanoma. The error bar indicates standard error of mean (SEM) of triplicate qPCR reactions.*

4.3 Analysis of exon 4/5 splice variant expression in human drug-resistant BRAF V600E melanoma cells treated with Vemurafenib.

In the comparison between expression of drug-resistant melanoma cells treated with (*Vemurafenib*$^{+}$*)* and without (*Vemurafenib*$^{-}$*)* additional drug treatment, the calibrator remains the exon 4/5 expression of the Donor 4 melanocytes. A significant 9.97-fold and 56.57-fold increase in expression of both exon 4/5 and exon 4/5 with insert was observed in *Vemurafenib*$^{+}$ cells compared to *Vemurafenib*$^{-}$ cells (P = 0.0202; P = 0.0050, respectively) (Fig. 14). Exon 4/5 with insert was shown to have a significantly lower expression by 25.71 fold in *Vemurafenib*$^{-}$ cells compared to exon 4/5 (P = 0.0001).

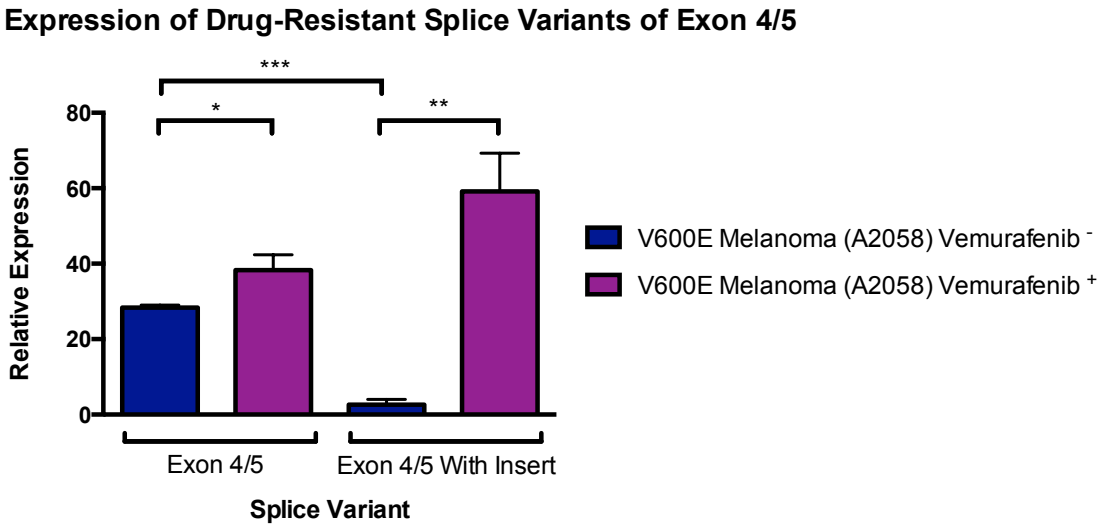

Figure 14: *RT-qPCR analysis of splice variants on multiple human drug-resistant samples. The relative expression of the reference isoform (exon 4/5) and the variant isoform (exon 4/5 with insert) was analyzed in melanocytes and BRAF V600E melanoma. The error bar indicates standard error of mean (SEM) of triplicate qPCR reactions.*

4.4 Combined analysis of exon 4/5 splice variant expression human melanocytes, BRAF V600E melanoma cells, and drug-resistant BRAF V600E melanoma cells.

This graph includes the expression of exon 4/5 and exon 4/5 with insert in all of the human samples. In comparison to the reference melanocyte, there was a significant increase in expression of exon 4/5 observed in BRAF V600E melanoma cells (see Section 4.2) as well as in *Vemurafenib*$^{-}$ and *Vemurafenib*$^{+}$ drug-resistant BRAF V600E melanoma cells by 27.34 fold and 37.30 fold, respectively ($P < 0.0001$; $P = 0.0058$, respectively) (Fig. 15). In the same conditions, exon 4/5 with insert also increased in BRAF V600E melanoma cells as well as by 58.18 fold in *Vemurafenib*$^{+}$ drug-resistant BRAF V600E melanoma cells ($P = 0.0045$).

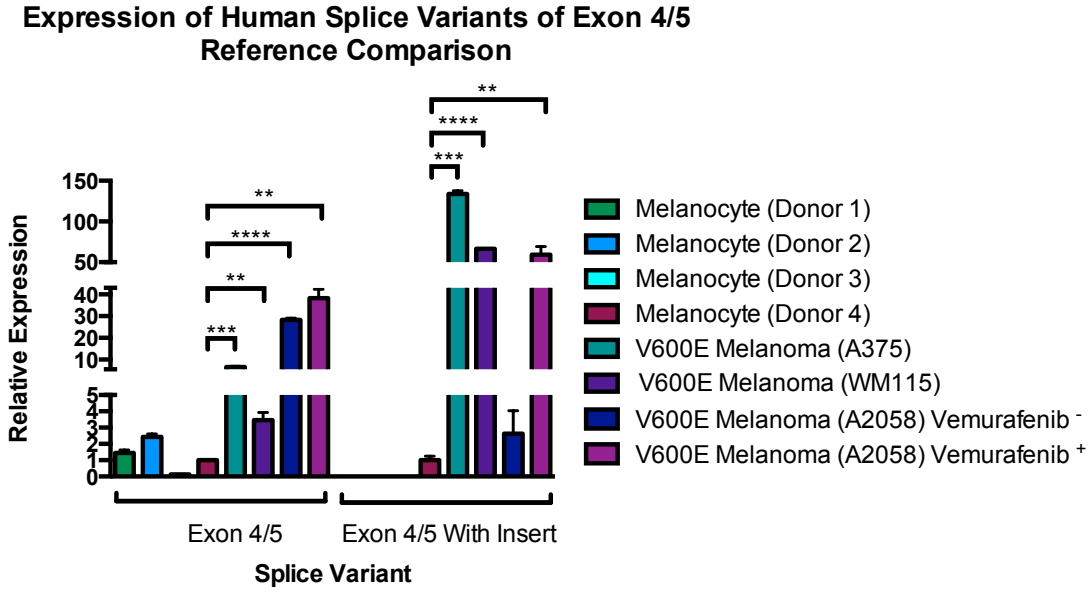

Figure 15: *RT-qPCR analysis of splice variants on multiple human samples. The relative expression of the reference isoform (exon 4/5) and the variant isoform (exon 4/5 with insert) was analyzed in melanocytes and BRAF V600E melanoma. The error bar indicates standard error of mean (SEM) of triplicate qPCR reactions.*

4.5 Combined analysis of exon 4/5 splice variant expression human melanocytes, BRAF V600E melanoma cells, and drug-resistant BRAF V600E melanoma cells with splice variant comparison.

This graph includes the relative expression of exon 4/5 and exon 4/5 with insert in all of the human samples. The expression of exon 4/5 with insert was undetected in melanocyte Donors 1, 2, and 3 while a low expression of exon 4/5 was observed in all melanocyte donors ($P = 0.0005$; $P < 0.0001$; $P = 0.0027$, respectively) (Fig. 16). A significantly higher expression of exon 4/5 with insert compared to exon 4/5 was observed in both BRAF V600E melanoma cells (A375 and WM115) by 127.3 fold and 62.95 fold, respectively ($P < 0.0001$; $P < 0.0001$, respectively). Additionally, there was a 25.71 fold lower expression of exon 4/5 with insert compared to exon 4/5 in *Vemurafenib*$^{-}$ BRAF V600E drug-resistant cells ($P = 0.0001$).

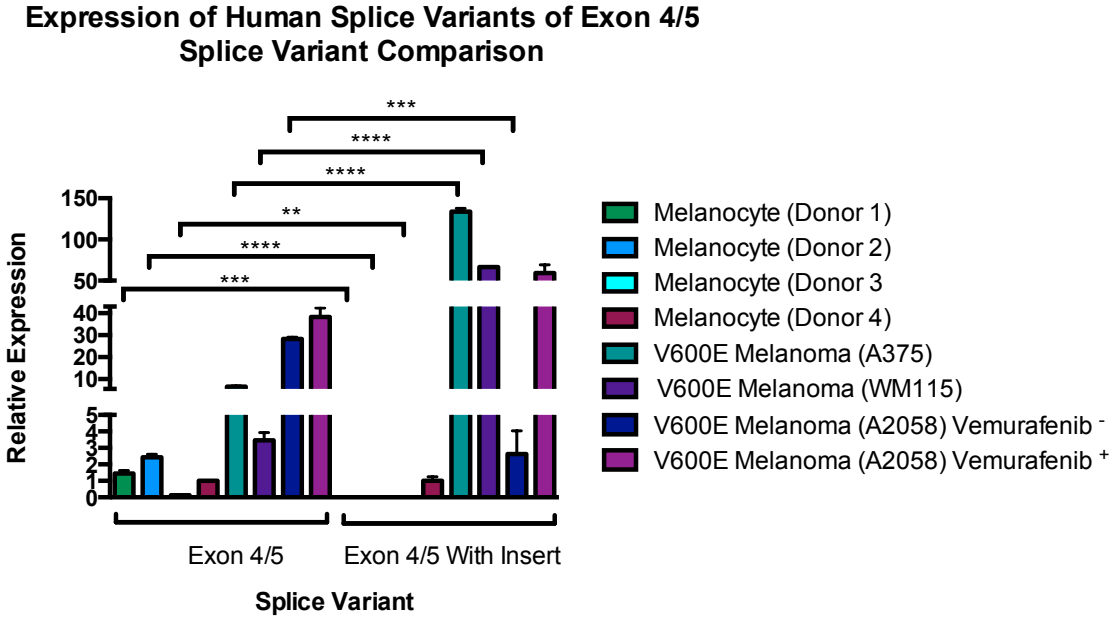

Figure 16: *RT-qPCR analysis of splice variants on multiple human samples. The relative expression of the reference isoform (exon 4/5) and the variant isoform (exon 4/5 with insert) was analyzed in melanocytes and BRAF V600E melanoma. The error bar indicates standard error of mean (SEM) of triplicate qPCR reactions.*

4.6 Analysis of exon 15/16 splice variant expression in mouse melanocytes and mouse BRAF V600E melanoma cells.

The melanocyte sample of the wild-type splice variant, exon 15/16, was chosen to be the calibrator for the same reasons as previously mentioned for exon 4/5 (see Section 4.1). A significant 0.60-fold decrease in expression of exon 15/16 and 0.43-fold decrease in exon 13/16 was observed in BRAF V600E melanoma cells compared to melanocytes ($P = 0.0034$; $P = 0.0007$, respectively) (Fig. 17). Expression of exon 13/16 was 0.17-fold higher than exon 15/16 in BRAF V600E melanoma cells ($P = 0.0229$).

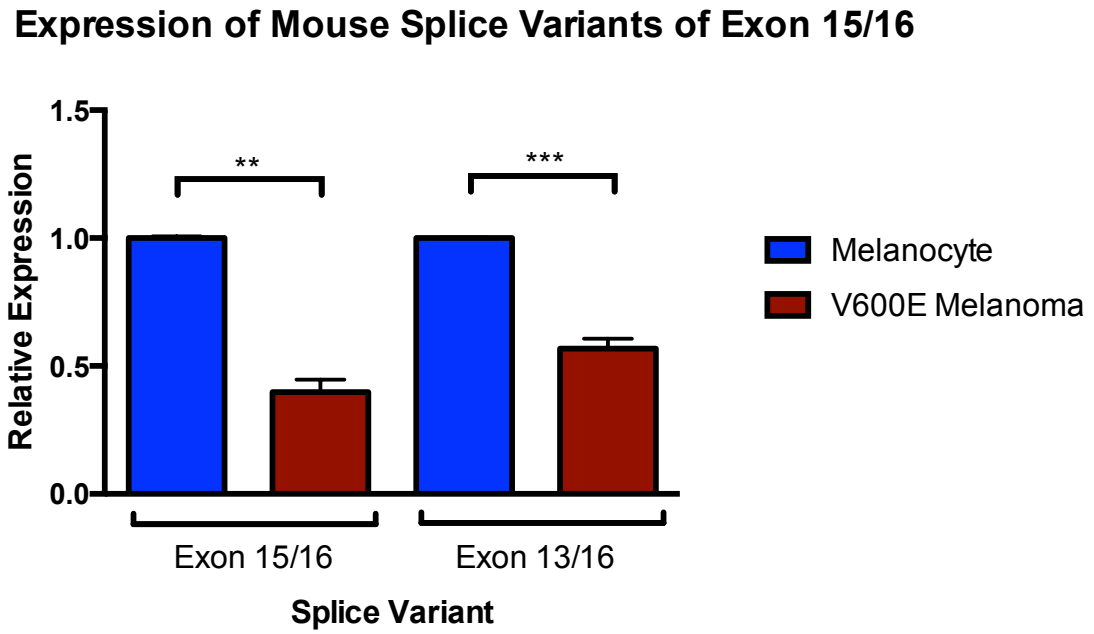

Figure 17: *RT-qPCR analysis of splice variants on multiple mouse samples. The relative expression of the reference isoform (exon 15/16) and the variant isoform (exon 13/16) was analyzed in melanocytes and BRAF V600E melanoma. The error bar indicates standard error of mean (SEM) of triplicate qPCR reactions.*

4.7 Analysis of exon 15/16 splice variant expression in human melanocytes and human BRAF V600E melanoma cells.

Although any of the melanocyte donors would represent a benign melanocyte in a healthy patient and act as an acceptable calibrator, for consistency with previous human sample analysis, melanocytes of Donor 4 for exon 15/17 was used (see Section 4.2). A significant 2.30-fold increase in expression of exon 15/16 was observed in BRAF V600E melanoma cells (A375) compared to reference melanocytes ($P = 0.0398$) (Fig. 18) and whereas a 5.70-fold and 4.93-fold increase of exon 13/16 is observed in both cell lines (A375 and WM115) ($P = 0.0002$; $P < 0.0001$, respectively). Exon 13/16 showed 3.40-fold higher expression than exon 15/16 in the A375 cell line of BRAF V600E melanoma cells ($P = 0.0069$).

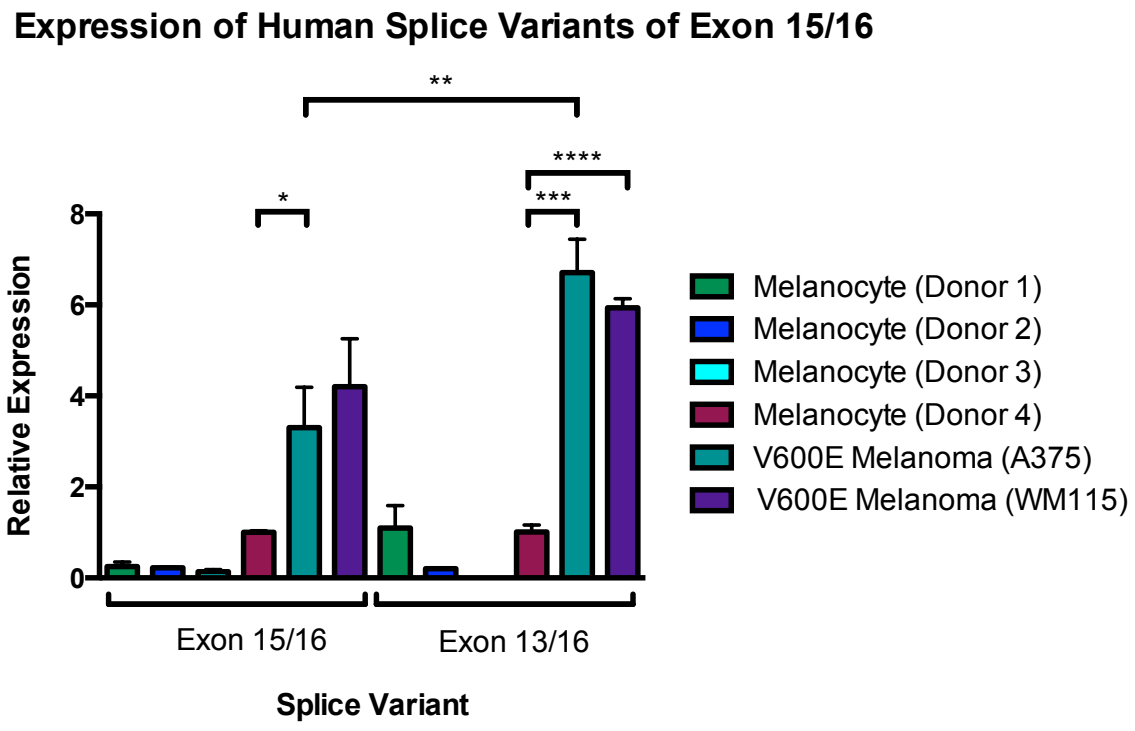

Figure 18: *RT-qPCR analysis of splice variants on multiple human samples. The relative expression of the reference isoform (exon 15/16) and the variant isoform (exon 13/16) was analyzed in melanocytes and BRAF V600E melanoma. The error bar indicates standard error of mean (SEM) of triplicate qPCR reactions.*

4.8 Analysis of exon 15/16 splice variant expression in human drug-resistant BRAF V600E melanoma cells treated with Vemurafenib.

The calibrator for this analysis remains the human melanocytes of Donor 4 of exon 15/16. A significant increase in expression of both exon 15/16 and exon 13/16 was observed in *Vemurafenib*$^{+}$ cells compared to *Vemurafenib*$^{-}$ BRAF V600E melanoma cells by 75.68 fold and 28.97 fold, respectively (P = 0.0032; P < 0.0001, respectively) (Fig. 19). A significantly 16.45-fold higher expression of exon 13/16 compared to exon 15/16 was observed in *Vemurafenib*$^{-}$ cells (P <0.0001) and a 30.26-fold lower expression in *Vemurafenib*$^{+}$ cells (P = 0.0120).

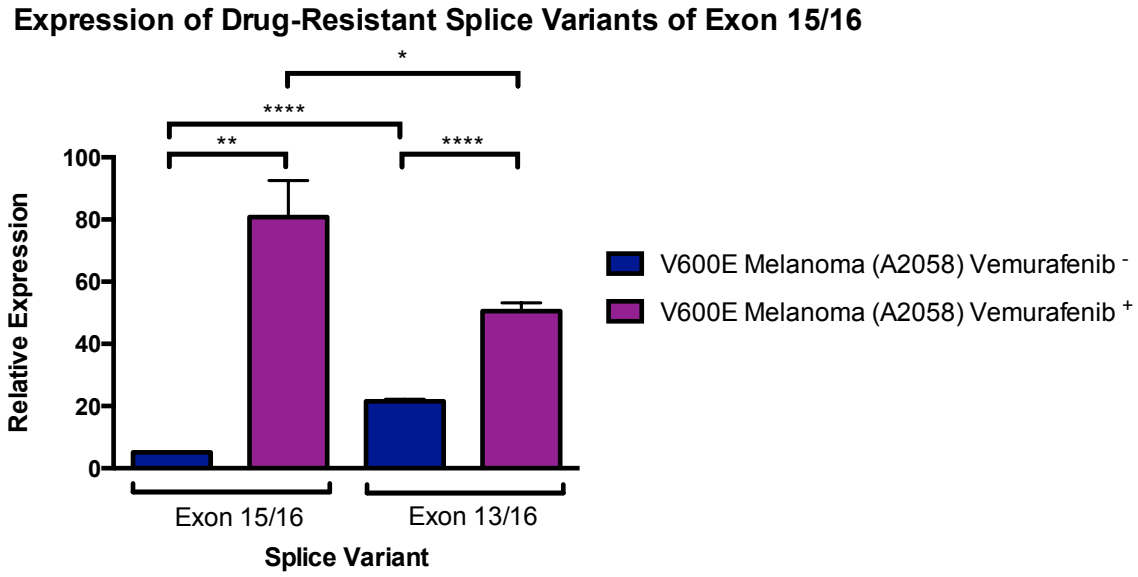

Figure 19: *RT-qPCR analysis of splice variants on multiple human drug-resistant samples. The relative expression of the reference isoform (exon 15/16) and the variant isoform (exon 13/16) was analyzed in melanocytes and BRAF V600E melanoma. The error bar indicates standard error of mean (SEM) of triplicate qPCR reactions.*

4.9 Combined analysis of exon 15/16 splice variant expression in human melanocytes, BRAF V600E melanoma cells, and drug-resistant BRAF V600E melanoma cells.

This graph includes the relative expression of exon 15/16 and exon 13/16 with insert in all human samples and illustrates a comparison between the reference melanocytes and all of the cell lines of BRAF V600E melanoma. A significant increase in expression of exon 15/16 was observed in BRAF V600E melanoma (see Section 4.7) as well as *Vemurafenib*$^{-}$ and *Vemurafenib*$^{+}$ drug-resistant BRAF V600E melanoma cells by 4.12 fold and 79.80 fold, respectively ($P = 0.0002$; $P = 0.0028$, respectively) (Fig. 20). Similarly, a significant increase in expression of exon 13/16 was observed in BRAF V600E melanoma cells (see Section 4.7) as well as *Vemurafenib*$^{-}$ and *Vemurafenib*$^{+}$ drug-resistant BRAF V600E melanoma cells by 20.56 fold and 49.53 fold, respectively ($P < 0.0001$; $P < 0.0001$, respectively).

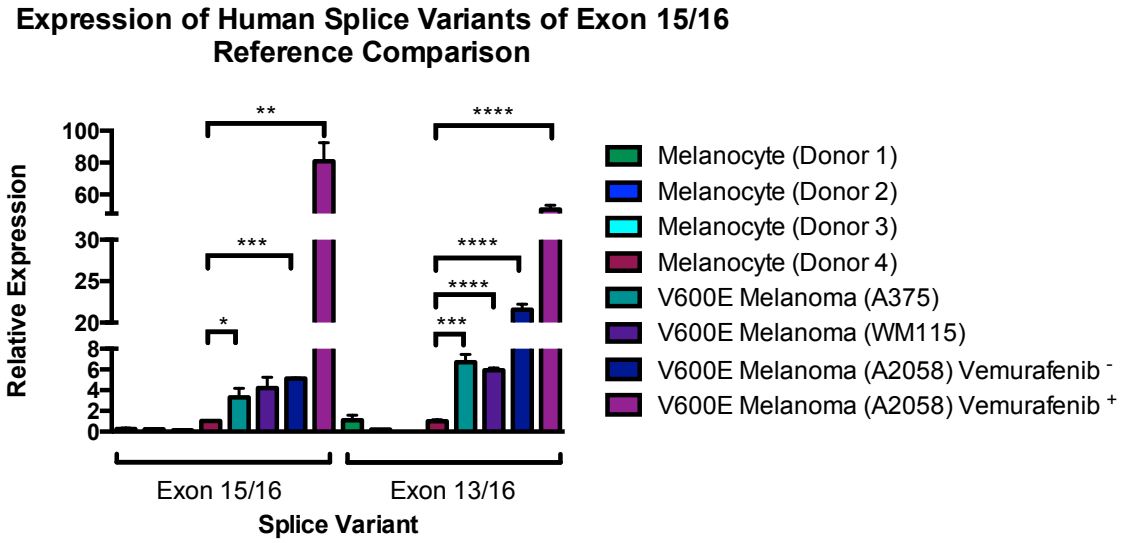

Figure 20: *RT-qPCR analysis of splice variants on multiple human samples. The relative expression of the reference isoform (exon 15/16) and the variant isoform (exon 13/16) was analyzed in melanocytes and BRAF V600E melanoma. The error bar indicates standard error of mean (SEM) of triplicate qPCR reactions.*

4.10 Combined analysis of exon 15/16 splice variant expression human melanocytes, BRAF V600E melanoma cells, and drug-resistant BRAF V600E melanoma cells with splice variant comparison.

This graph includes the relative expression of exon 15/16 and exon 13/16 with insert in all human samples and illustrates a comparison between splice variants in singular cell lines. We observed a higher expression of exon 13/16 compared to exon 15/16 in BRAF V600E melanoma cells (see Section 4.7) as well as in *Vemurafenib*$^{-}$ BRAF V600E melanoma cells by 16.45 fold ($P < 0.0001$) (Fig. 21). Whereas in *Vemurafenib*$^{+}$ BRAF V600E melanoma cells, there was a 30.26-fold higher expression of exon 15/16 compared to exon 13/16 ($P = 0.0120$).

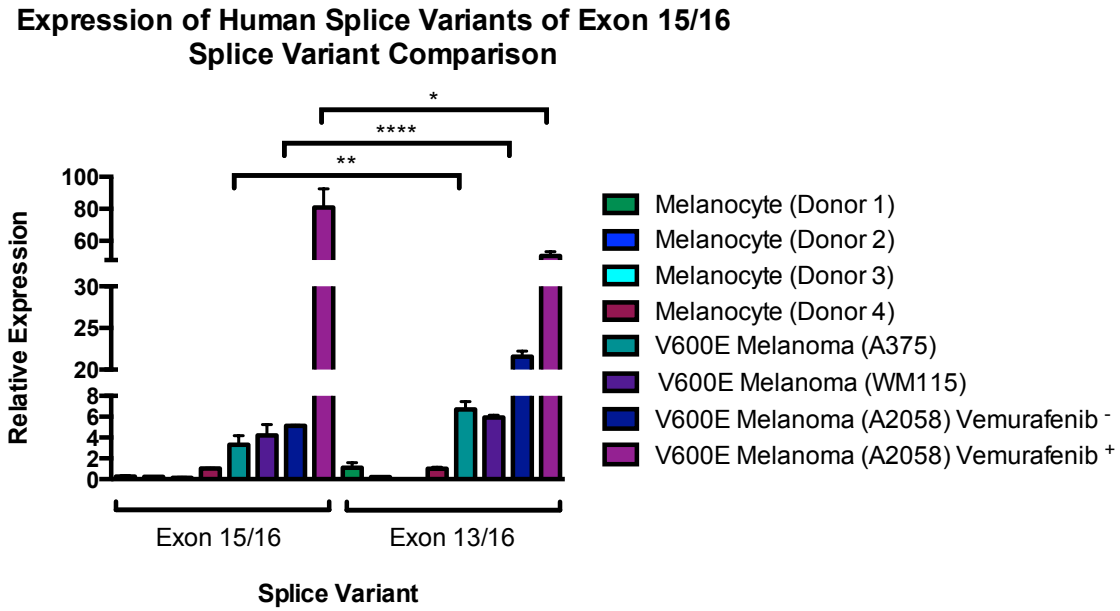

Figure 21: *RT-qPCR analysis of splice variants on multiple human samples. The relative expression of the reference isoform (exon 15/16) and the variant isoform (exon 13/16) was analyzed in melanocytes and BRAF V600E melanoma. The error bar indicates standard error of mean (SEM) of triplicate qPCR reactions.*

5. DISCUSSION

BRAF is frequently subjected to alternative splicing and the novel splice variants discovered in Dr. Indra's lab were found to be expressed in melanocytes and non-resistant and drug-resistant BRAF V600E melanoma. The nucleotide insertion between exon 4 and 5 and the deletion of exon 14 and 15 alters the protein sequence of BRAF which may then lead to gain of function, loss of function, or altered specificity of the protein's function.[22] The results of RT-qPCR, while not indicative of the splice variants' exact role in altering BRAF's activity, is suggestive of the preferential splicing that occurs in BRAF V600E-mutated melanoma.

The 3-nucleotide insert between exon 4/5 (exon 4/5 with insert) occurs in the Ras-binding domain. This would seemingly disrupt the ability of Ras to bind to Raf and discontinue the sequential activation of the MEK/ERK pathway. However, the BRAF V600E mutation alone may activate the pathway through its negative charges mimicking phosphorylation.[4] Thus, exon 4/5 with insert could have a role in enhancing BRAF's kinase activity despite its disruption in binding to Ras.

In closer analysis of the expression of exon 4/5 and exon 4/5 with insert in human samples (Fig. 13), only the melanocytes of Donor 4 showed expression of exon 4/5 with insert. The other three melanocyte donors had undetected expression. This seems plausible because these samples are benign, and we believe exon 4/5 with insert is a mechanism of benign melanocytes to transform into malignant melanomas. Donor 4 was the only melanocyte sample to have consistently moderate expression of every splice variant in melanocytes and BRAF V600E melanoma. This may be due to the collection of the Donor 4 sample from benign nevi with melanocytes harboring oncogenic

mutations in a growth-arrested phase, rather than a transition into malignancy. The presence of the V600E mutation in BRAF in this sample is possible because this mutation has been found in nevi that are senescent for many years.[22]

In RT-qPCR analysis of the mouse samples (Fig. 12), we saw a pattern of decreased expression in both exon 4/5 and exon 4/5 with insert in BRAF V600E melanoma compared to melanocytes. This is the opposite trend we observed in human samples which suggests that there may be a different preferential splicing mechanism between the species. However, we did see a higher expression of exon 4/5 with insert compared to exon 4/5 when looking at only the V600E melanoma cell line and no change when looking at the melanocytes. This alludes to potential preferential splicing in which exon 4/5 with insert enhances BRAF activity; however, analysis on human samples would provide stronger evidence of this role.

In human samples (Fig. 13), Exon 4/5 with insert was completely undetected in three of the four melanocyte donors while increasing by over 100-fold in BRAF V600E melanoma. A causative role of exon 4/5 with insert in tumor progression is probable because of its striking upregulation in only the melanoma cells. By analyzing the lesser-marked upregulation of exon 4/5, it seems that it's not just BRAF protein expression that is upregulated along with all of its splice variants. Rather, exon 4/5 with insert is preferentially expressed over the wild-type exon 4/5.

However, in drug-resistant BRAF V600E melanoma cells (Fig. 14, 15), increased expression of exon 4/5 with insert was not as significant as expected, and it was especially low in the sample without additional Vemurafenib treatment. One possible explanation is that the drug-resistant cells were received after a period of time when the

resistance could have weakened. This lack of resistance may have occurred because of the loss of microenvironment of the cells from the epidermis to the petri dish of cell culture. Immediate analysis of the resistant cells after additional treatment with Vemurafenib resulted in exon 4/5 increasing slightly while exon 4/5 with insert increased very significantly (56 fold). This preferential splicing suggests a potential role in providing resistance to a Vemurafenib-treated melanoma cell.

The splice variant with deletion of exon 14 and 15 (exon 13/16) occurs in the kinase domain of BRAF. The splice variant is most likely kinase dead and thus would not activate the pathway in an enhanced-kinase activity manner. However, the pattern of increased expression in BRAF V600E melanoma suggests that exon 13/16 is preferentially spliced. It could be assumed that if the splice variant plays a role in tumor progression, it would provide this with a different mechanism.

Similar results occurred in the mouse samples as with exon 4/5 and exon 4/5 with insert. The significant decreased expression of exon 15/16 and exon 13/16 in BRAF V600E melanoma compared to melanocytes was unexpected (Fig. 17). It would suggest that these splice variants are not preferentially expressed in this type of melanoma, however the increase of exon 13/16 compared to exon 15/16 in the V600E is worth noting. The melanocytes did not exhibit this increase in expression. This brought interest in further investigation of this splicing pattern in humans.

In human samples, a very significant increase in expression of exon 13/16 was observed in BRAF V600E melanoma cells compared to melanocytes (Fig. 18, 19). This occurrence was much greater in significance than the increase of exon 15/16 in BRAF V600E melanoma cells compared to melanocytes, which is additionally supported by the

marked difference in comparison between the splice variants in one cell line of BRAF V600E melanoma cells. The pattern of expression of exon 13/16 suggests preferential splicing in this type of melanoma.

Exon 13/16 could also have a role in drug resistance due to the significant increases observed in BRAF V600E melanoma cells compared to melanocytes (Fig. 20). The comparison between exon 15/16 and exon 13/16 in the drug-resistant samples do not exhibit the potential role in resistance of this aberrant exon 13/16 splice variant. However, when comparing exon 13/16 expression in drug-resistant melanoma compared to melanocytes, there is marked upregulation.

6. CONCLUSIONS

Alternative splicing is frequently investigated in cancer progression because differential expression of splice variants may play a role in the oncogenic characteristics of a cancer cell.[15] This study has successfully identified novel splice variants (exon 4/5 with insert and exon 13/16) in non-resistant and drug-resistant melanoma with the BRAF V600E mutation. Differential expression of these gene products between benign melanocytes and BRAF V600E melanoma was confirmed by RT-qPCR. A general trend of increased expression of exon 4/5 with insert and exon 13/16 in BRAF V600E melanoma was observed in this study. Preferential expression of these splice variants may be a regulatory mechanism to promote MEK/ERK signaling and thus tumor progression.[15] However, evidence of their involvement in upregulating the kinase activity of BRAF V600E is still lacking. Functional evaluation of these novel splice variants would provide deeper understanding of the molecular mechanism of melanoma cancer caused by BRAF V600E mutation.[21]

Recently there has been an increased incidence of melanoma cases in the United States.[2] Late stages of melanoma have a poor prognosis and are not sensitive or become resistant to available treatments.[5] This lack of treatment calls for new treatments, and BRAF may provide an option. BRAF V600E is mutated over 50% of melanoma cases and thus it presents an ideal target for therapy.[8] Raf-1, another member of the Raf family, has traditionally been the primary target for anti-cancer agents such as antisense compounds, kinase inhibitors, and dominant interfering DNA constructs.[4] However, drugs targeting Raf-1 do not directly inhibit MEK/ERK activity, rather they inhibit Raf-1's specific role in cell survival. BRAF, on the other hand, has a direct relationship

with MEK/ERK activation and targeting this protein would be effective in inhibiting this pathway altogether.[4] One possible method would be to develop agents that target splice variants that increase the kinase activity of BRAF. By disrupting the BRAF/MEK/ERK pathway, tumor progression would be halted because of its mediation of many characteristics of cancer.

The role of BRAF in melanoma and other cancers needs to be more fully understood. Further research must be done the role of splice variants on BRAF expression, structure, and regulation of activation in order to know exactly its role in MEK/ERK activation and tumor progression.[4] Once the contribution of BRAF splice variants to tumor progression and malignancy is established, we can then develop effective anti-cancer therapies. Future directions to improve our understanding of these novel splice variants include measuring their expression in melanoma samples with a non-V600E BRAF mutation. We could then determine if these splice variants are specific towards this mutation or occur in other cases of melanoma. We could also design antibodies towards these splice variants and perform immunoprecipitation to further analyze the regulation of alternative splicing of BRAF. Further investigation to determine the splice variants' function in modulating BRAF's kinase function is also needed.

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