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Review Article

Arg393his Antithrombin Hanoi Deficiency and Melanoma Associated with *BRAF* Mutation: Potential Molecular Link Via Epistasis and Cross-Talk of Signaling Pathways

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Abstract

Antithrombin (AT), a pleiotropic protein with multifaceted roles in intracellular functions outside of its anticoagulant function such as in host defense, inflammation, anti-angiogenesis and tumorigenesis. *BRAF* gene, a proto-oncogene, serine/threonine kinase, encodes a protein called BRAF, which regulates the signal transduction serine/threonine-specific protein kinase in the RAS/RAF/MEK/ERK signaling pathway. Mutations in *BRAF* gene can be inherited and cause some developmental disorders such as cardiofaciocutaneous (CFC) syndrome, Noonan syndrome (NS), Costello syndrome, and LEOPARD syndrome (collectively known as RASopathies) as well as birth defects and/or acquired, appeared later in life, and cause cancers. This article demonstrates (a) for the first time, a potential molecular link via epistasis and cross-talk of signaling pathways in which the mutation V600E (T1799A) of the *BRAF* gene acted as a "modifier" gene in the development of melanoma from an Arg393His antithrombin Hanoi deficiency's patient; and (b) as perspectives, exploration of the potential treatments for cancer.

Keywords: Antithrombin, Arg393His antithrombin Hanoi, *BRAF* mutation, Epistasis, Expression vectors via glycosylphosphatidylinositol, Melanoma, Modifier gene, RAS/RAF/MEK/ERK signaling pathway, Sonogenetics

Introduction

Human antithrobin (AT), a heparin-binding serpin (heparin cofactor), is a serine protease inhibitor. It is encoded via the *SERPINC*1 (serpin peptidase inhibitor, clade C, member 1) gene, located in chromosome 1 (q23-25) and has 7 exons. It is a glycoprotein synthesized in the liver and has a molecular weight of 58200 Daltons with 432 amino acids [1]. Among blood clotting disorders, AT deficiency (although rare in the general population: the prevalence of heterozygous AT deficiency varies between 0.02% and 0.2% [2]) is associated with the highest thrombotic events in veins (with venous thromboembolism, VTE, including deep venous thrombosis, DVT, and pulmonary embolism, PE) or in arteries, which are the underlying cause of strokes and heart attacks (myocardial infarction, MI) as well as peripheral artery disease (PAD) [1]. In addition to being an anticoagulant, AT also possesses potent anti-inflammatory, anti-angiogenesis and tumorigenesis activities. So that AT can be defined as a pleiotropic protein [3,4]. Concerning cancer, melanoma is a malignant proliferation of melanocytes - the cells that produce melanin (skin pigment). Melanoma is the most aggressive type

of skin cancer because it grows so quickly. While primarily found on sun-exposed skin, it can also develop from moles or in "hidden" areas like the palms, between the toes, soles, genital area, eyes, or mucous membrane. Early detection is vital, as melanoma can spread (metastasizes) first into nearly lymph nodes before going to nearby tissues and distant organs (such as the liver, lungs, bone, or brain, or to skin in another part of the body) if left untreated [5,6]. According to a report by the World Health Organization (WHO), about 48,000 people die of melanoma every year [7]. The discovery of somatic mutation V600E (Val600Glu), a mutation at T1799A in exon 15 of *BRAF* (a proto-oncogene) in 2002 by Davies et al. [8] was a major breakthrough in cancer research for targeted therapy. Note that a proto-oncogene is a normal gene involved in cell growth and proliferation or inhibition of apoptosis (self-destruction of cells). A proto-oncogene could become an oncogene due to a relatively small modification of its original function such as (a) missense mutation, insertion, deletion, or duplication; (b) increased expression through mutations in the promoter region, for example; (c) epigenetic modifications such as epigenetics describes factors beyond the genetic code such as gene-gene-interactions: epistasis, and/or gene-environment interactions rather than genetic alterations; or (d) different chemical compounds that can be linked to genetic material such as DNA or RNA may have an impact on genes. Oncogenes have the potential to cause normal cells to become cancerous when they become activated [9]. This V600E (Val600Glu) somatic mutation accounts for greater than 90% of the observed mutations in *BRAF* gene in which 66% of melanomas, whereas 30% of other tumors, such as colorectal cancer (15%), lung cancer (12%), thyroid cancer (3%) [8,10]. Importantly, this *BRAF* gene is located at a crossroads of the linear RAS-RAF-MEK-ERK signal transduction cascade, also referred to as the mitogen-activated protein kinase (MAPK) pathway, and plays a central role for cell proliferative responses. Abnormal activation of this MAPK pathway is implicated in the pathology of diverse human diseases including cardiovascular diseases, neurological and developmental disorders such as Alzheimer's disease (AD), Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS), autism spectrum disorders, RASopathies, and various types of cancers, and is now an attractive target for therapeutic strategies of cancer [10-13]. This article will focus on *BRAF* and demonstrate (a) for the first time, a potential molecular link via epistasis and cross-talk of signaling pathways in which the mutation V600E (T1799A) of the *BRAF* gene acted as a "modifier " gene in the development of melanoma from an Arg393His antithrombin Hanoi deficiency's patient; and (b) as perspectives, exploration of the potential treatments for cancer.

Case history of AT deficiency of the proband and his family history from Arg393His mutation in AT-Hanoi overview

For details, please refer to Refs. # 1,4. Briefly, the proband (II-2) has had a heterozygous point mutation Arg393His (R393H) at exon 7 of AT (named as AT-Hanoi for the city in Viet Nam where the proband (II-2) was born), which is associated with the highest thrombotic events. Besides the presence of cerebrovascular accident, CVA, (stroke): encephalomalacia/gliosis at age 23 (with no specific treatments, and this stroke is gone by its own), DVT of the leg, and kidney cancer at age 42, he is currently (73 year-old) very well (under long-term treatment with warfarin of 3mg/day to prevent blood clots and maintaining an international normalized ratio (INR) of 2 to 3 as well as with atorvastatin of 10mg/day to prevent high levels of cholesterol and atherosclerosis), free of cancer (because after surgery to remove the kidney affected by cancer and over 31 years of surveillance, there is no detectable cancer in his body means that cancer cells are gone), and thrombosis. Note that at age 42, the proband (II-2) was diagnosed with kidney cancer just after two weeks of treatment with warfarin (1mg/day) for DVT, and after the surgical removal of the left kidney affected by cancer, he continued the treatment with warfarin only (1mg/day). No specific cancer treatment such as chemotherapy, radiotherapy, etc. was applied after this surgery. In addition, the factor VIII activity (measured from blood sample of the proband II-2) is high: 224% compared to the standard range of 55-140%, and there are no mutations detected from all exons and flanking intronic sequences of the tumor suppressor protein p53 (*TP53*) and the homologue of the murine double minute 2 protein (*HDM2*) genes from DNA isolated from whole peripheral blood of the proband (II-2) (data not shown). However, the most important event occurred recently in this family concerns the son (III-4) (39 years old and carrier of the heterozygous Arg393His of AT Hanoi) of the proband (II-2): a skin cancer of melanoma developed from a mole on the back and has spread to the lymph nodes (stage III) from this son (Figure 1). After surgical removal of the mole and three lymph nodes affected by cancer, he was under the immune checkpoint inhibitor (ICI) therapy (Pembroliumb) given by intravenous infusion in which common adverse reactions such as eczema, and poliosis have been observed. After three rounds of treatment with this ICI therapy within two months, he was under the combination treatment with Dabrafenib (Tafinlar) for the V600E (T1799A) mutation of the *BRAF* (proto-oncogene *BRAF*) gene [8] and Trametinib (Mekinist) (inhibitor of MEK, mitogen-activated protein kinase enzymes) for one year to prevent melanoma from coming back. Note that the somatic missense mutations in the *BRAF* gene have been found in 66% of malignant melanoma and at lower frequency in a wide range of human cancers [8]. He is actually under the positron emission tomography (PET)

scan for following up care to check for cancer recurrence or metastasis. It is important to note herein that all members of this family such as: the mother (I-2), carrier of AT deficiency, was asymptomatic and deceased at age 97; the proband's brother (II-3) (71 year-old) showed the presence of heterozygous Arg393His mutation, had suffered DVT of the leg and mesenteric thrombosis at age 50 and one of the two his children (III-6) showed the presence of heterozygous Arg393His mutation, is actually no symptoms of AT deficiency; the proband's brother (II-4) (69 year-old) showed the presence of heterozygous Arg393His mutation had suffered DVT of the leg at age 60 (Figure 1). Note that like the proband II-2, all patients with symptoms of AT deficiency such as II-3, and II-4 were under long-term treatment with warfarin to prevent blood clots and up to present, there is no cancer development observed from these patients. Otherwise, the son (III-4) as well as all members of this family are non-smoking, non-alcoholic, and are not frequently exposed to the sun.

Overview of BRAF in the RAS-RAF-MEK-ERK signal transduction pathway III

1. RAS-RAF-MEK-ERK signal transduction pathway

A signaling pathway is a process by which a chemical or physical signal is transmitted through a cell as a series of molecular events and via this process a cell interacts with itself, other cells, and the environment. When signaling pathways interact with one another they form networks, which allow cellular responses to be coordinated, often by combinatorial signaling events [14]. Extracellular signals (like hormones or growth factors) can bind to cell receptors triggering intracellular signal transduction, at the molecular level, including changes in the transcription, translation of genes or post-translational and conformational changes in proteins to regulate cellular functions such as growth, metabolism, or gene expression [15,16]. Concerning the RAS-RAF-MEK-ERK signal transduction pathway, it is the first and well-defined mitogenic pathway that couples signal from cell surface receptors to transcription factors, which regulate many fundamental cellular processes such as cell proliferation and survival, differentiation, apoptosis, and secretion, etc. For details, please refer to Refs. # 10-13. Briefly, this signal transduction pathway includes many proteins, such as mitogen-activated protein kinases (MAPKs) (a kinase is an enzyme that catalyzes the transfer of phosphate groups to substrates. This process is known as phosphorylation), originally called extracellular signal-regulated kinases (ERKs), which communicate by adding phosphate group to a neighboring protein (phosphorylating it), thereby acting as an "on" or "off" switch. When one of the proteins in the pathway is mutated, it can become stuck in the "on" or "of" position, a necessary step in the development of many cancers. All components of the MAPK/ERK pathway were first discovered in cancer cells, and drugs that reverse the "on" or "of" switch are being investigated as cancer treatments. This pathway exists in all eukaryotes (Figure 2). In many cell types, activation of this pathway promotes cell division, and many forms of cancer are associated with aberrations in it. Many proteins that are phosphorylated by MAPK such as the interacting protein kinases (MNKs, with two subtypes MNK1 and MNK2, and are serine/threonine kinases), which regulate the initiation of translation through phosphorylation of eukaryotic initiation factor 4E (eIF4E, plays a central role in translation initiation and is involved in regulating protein synthesis by directing ribosomes to the 5'-cap of mRNA, thereby facilitating efficient protein synthesis. Its mRNA cap-binding activity influences a range of biological processes and diseases state, making it an important target for therapeutic development, particularly in disorders characterized by aberrant protein production) and are shown in Figure 3. While the phosphorylation of eIF4E is necessary for oncogenic transformation, the kinase activity of MNKs seems dispensable for normal development. For this reason, the development of inhibitors of MNKs could represent an ideal mechanism-based and nontoxic therapeutic strategy for cancer treatment. In anyway, such series of kinases provide opportunities for feedback regulation and signal amplification.

RAS (EC 3.6.5.2), short for rat sarcoma virus, which is a family of related proteins family that are expressed in all animal cell lineages and organs. This RAS family is the first oncogene discovered. Usually, RAS is located on the inner leaflet of the plasma membrane (on the cytoplasmic side of the plasma membrane). There are three main isoforms of *RAS* gene in humans: *HRAS, KRAS, and NRAS* and they are the most common oncogenes in human cancer. All RAS protein family members belong to a class of protein called small GTPases, a large family of hydrolase enzymes that bind to the nucleotide guanosine triphosphate, GTP, and hydrolyze it to guanosine diphosphosphate, GDP. These enzymes are active when bound to GTP and inactive when bound to GDP. RAS acts as a branch point in signal transduction because it orchestrates the activity of multiple signaling pathways to regulate diverse cellular function. Mutations in RAS genes are very common, being found in 20% to 30% of all human tumors and can lead to the production of permanently activated RAS proteins, which can cause unintended and overactive signaling inside the cell, even in the absence of incoming signals.

RAF (RAF family kinases: EC 2.7.11.25) is an acronym for rapidly accelerated fibrosarcoma. RAF kinases are a family of three serine/threonine-specific protein kinases that are related to retroviral oncogenes. The three RAF kinase family members are: ARAF, BRAF, and CRAF (CRAF is also called RAF-1). All RAF isoforms are cytosolic proteins. The genes for RAF proteins are located on chromosomes X, 7, and 3 for A, B, and CRAF, respectively. Activation of RAF kinases requires interaction with RAS-GTPases. RAF mutations (particularly in BRAF) have been found in 8% of all cancer types.

MEK (mitogen extracellular kinase: EC 2.7.12.2, functioning as mitogen-activated protein kinase kinase, MAPKK or MAP2K) has two subtypes MEK 1 and MEK 2, and are dual-specificity kinases that phosphorylate both serine/threonine and tyrosine residues. MEK proteins are directly phosphorylated and activated by RAF kinases.

ERK (extracellular signal-regulated kinases: EC 2.7.11.24, functioning as mitogen-activated protein kinase, MAPK or MAP kinase) has two subtypes ERK1 and ERK 2. Once activated, MEK in turn activates ERK by phosphorylation of ERK on specific threonine and tyrosine residues. When the signaling pathway is inactive, MEK anchors ERK in the cytoplasm. Active ERK can translocate into the nucleus, where it phosphorylates various transcription factors, such as Elk-1 (Elk-1 functions as a transcriptional activator, a protein that increases transcription of a gene or a set of genes. Elk-1 plays important roles in various contexts, including long-term memory formation, drugs addiction, AD, Down syndrome, breast cancer, and depression) and C-myc (a family of regulator genes and proto-oncogenes. C-myc, also sometimes referred to as MYC, was the first gene to be discovered in this family, due to homology with the viral v-myc: an avian virus, myelocytomatosis. In cancer, C-myc is often persistently expressed. This leads to the increased expression of many genes, some of which are involved in cell proliferation, contributing to the formation of cancer), thereby modulating gene expression related to cell proliferation and survival. The frequency of MEK mutations is low (~1%), though a few pathogenic mutations in ERK have been mentioned.

The RAS-RAF-MEK-ERK signal transduction pathway (Figure 3) is started by the RAS activation (RAS is normally present in the inactive GDP-bound form) via the binding of extracellular mitogen (a small bioactive protein or peptide that induces a cell to begin cell division, or enhances the rate of division: mitosis) such as the epidermal growth factor (EGF: extracellular ligand) to a cell surface receptor such as the epidermal growth factor receptor (EGFR). Binding of EGF to the EGFR activates the tyrosine kinase activity of the cytoplasmic domain of the receptor. The EGFR becomes phosphorylated on tyrosine residues. Docking proteins such as the growth factor receptor-bound protein 2 (GRB2) contain a Src homology 2 (SH2) domain that binds to the phosphotyrosine residues of the activated receptor. GRB2 binds to the guanine nucleotide exchange factor of son of sevenless (SOS) by way of the two Src homology 3 (SH3) domains of GRB2. When the GRB2-SOS complex docks to phosphorylated EGFR, SOS becomes activated. Activated SOS then promotes the removal of GDP from a member of the RAS subfamily (most notably H-RAS or K-RAS). The RAS protein can then bind GTP and become active. Apart from EGFR, other cell surface receptors that can activate this pathway via GRB2 include tropomyosin receptor kinase (Trk A/B), fibroblast growth factor receptor (FGFR) and platelet-derived growth factor receptor (PDGFR). However, EGFR is an important molecule involved in cancer biology and therapy. It is a growth factor-dependent receptor tyrosine kinase that is involved in a host of critical cellular responses such as growth, survival, and proliferation. Disorders in the EGFR gene are common in many types of cancers and several cancer therapies are based on EGFR-targeted antibodies in conjunction with radiation or chemotherapy. Activated RAS recruits RAF to the plasma membrane, where RAF undergoes complex phosphorylation events leading to its activation. This step requires multiple protein-protein interactions and conformational changes. The RAF kinase phosphorylates and activates a MEK (MEK1 or MEK2). The MEK phosphorylates and activates a MAPK. MAPK phosphorylates the 40S ribosomal protein S6 kinase (RSK). This activates RSK, which in turn, phosphorylates ribosomal protein S6 (mouse model studies have shown that phosphorylation of ribosomal protein S6 is involved in the regulation of cell size, cell proliferation, and glucose homeostasis). MAPK regulates the activities of several transcription factors. MAPK can phosphorylate C-myc MAPK phosphorylates and activates MNK, which in turn, phosphorylates cAMP response element-binding protein (CREB, it has a role in neuronal plasticity and long-term memory formation in the brain. CREB downregulation is implicated in the pathology of AD and increasing the expression of CREB is being considered as a possible therapeutic target for AD). MAPK also regulates the transcription of C- Fos gene (a proto-oncogene and transcription factor that plays an important role in many cellular functions and has been found to be overexpressed in a variety of cancers). By altering the levels and activities of transcription factors, MAPK leads to altered transcription of genes that are important for cell-division cycle.

The ERK kinase plays a central role within the RAS-RAF-MEK-ERK signal transduction pathway, exerting control over various facets of cellular metabolism in cancer cells. Then, when it comes to development of anticancer drugs, the following three key upstream regulators and ERK are aimed: RAS (upstream activator of RAF), RAF (direct effector of RAS and activator of MEK), MEK and ERK. Historical events of the discovery and development of the RAS-RAF-MEK-ERK pathway in health and diseases as well as anticancer drugs are summarized in [17] (Figure 4). Disregulation of this pathway is a common event in cancer as two components of this pathway, RAS and RAF, are proto-oncogenes, and are the most frequently mutated oncogenes in human cancers such as melanoma, papillary carcinoma, prostate cancer, colorectal cancer, etc. [10] (Figure 5). It is important to note that besides their established role in tumorigenesis, RAS, RAF proteins and the RAS-RAF-MEK-ERK signal transduction pathway have been shown to play key roles in various “normal” physiological cellular processes such as cellular metabolism, cell cycle progression, cell death, regulation of cardiomyocyte survival and growth, control of blood pressure, and neurological function [18-29].

2. BRAF Structure and Mutations in Cancer

This article will focus on *BRAF* due to (1) RAF kinase protein is phosphorylated and stimulated by activated RAS. Here, BRAF may be the primary target of oncogenic RAS (ARAF and CRAF appeared to be weakly stimulated by oncogenic RAS) [26]; (2) the main propose of RAF is to act as a mediator in the activation of MEK1/2 proteins that are directly upstream of ERK, and compared to ARAF, BRAF is a stronger inducer of MEK phosphorylation [26]; (3) various BRAF somatic mutations are quite common in cancers such as melanomas, colorectal cancer, lung cancer, and thyroid cancer. Based on the American Association for Cancer Research's (AACR) and Genomic Evidence Neoplasia Information Exchange (GENIE, version 15.1) dataset, BRAF alterations are observed in several human cancers with different frequencies [30] (Figure 6). Of these alterations, the somatic mutation V600E (Val600Glu) of *BRAF* gene [8] accounts for greater than 90% of the observed mutations in BRAF in which 66% of melanomas, whereas 30% of other tumors, such as colorectal cancer (15%), lung cancer (12%), thyroid cancer (3%) [8,10] (Figure 7); (4) the discovery of somatic mutation V600E (Val600Glu) of *BRAF* gene [8] was the major breakthrough in cancer research for targeted therapy. The effectiveness of a targeted agent depends on where in the pathway the activating mutant occurs and what other pathways the aberrant protein is channeling. Thus, it may be of greater benefit to target downstream in the BRAF pathway and MEK seems to be a logical target for targeted therapy [27]. However, although MEK is downstream of both RAS and RAF in the RAS-RAF-MEK-ERK signal transduction cascade, Solit and colleagues [21] showed that BRAF mutation, but not RAS mutation, could predict sensitivity to MEK inhibition, indicating the BRAF mutations are more reliant on MEK/ERK signaling than RAS mutants [21,27]; and (5) the presence of the V600E (Val600Glu) somatic missense mutation of *BRAF* in melanoma (stage III) observed from the son (III-4) (39 years old and carrier of the heterozygous Arg393His of AT Hanoi) of the proband (II-2) (Figure 1).

Note that *BRAF* (OMIM: 164757; EC 2.7.11.1; RAF family kinases: EC 2.7.11.25) is a proto-oncogene located on chromosome 7 (7q34) and encodes a protein called BRAF. The BRAF, an oncoprotein and a serine/threonine protein kinase, contains 766 amino acids, has a molecular weight of 84436 daltons, and is comprised of 18 exons with three regions (CR1-CR3) that are conserved across the RAF family members. CR1 and CR2 (in the N terminus) correspond to the regulatory domain and CR3 (in the C terminus) is the kinase domain [10,31] (Figure 8). CR1 (encoded by amino acids 150-290) contains the RAF-like RAS binding domain (RBD) and a cysteine-rich domain (CRD, an auto-inhibitor of the kinase domain), the second RAS-binding site. Residues 155-227 are the RBD, which binds to RAS-GTP's effector domain to release CR1 and halt kinase inhibition. Residues 234-280 comprise a phorbol ester/DAG-binding zinc finger motif (phorbol esters are the tetracyclic diterpenoids generally known for their tumor promoting activity; DAG: diacyl glycerol, activator of protein kinase C, which regulates different signal transduction pathways and other cellular metabolic activities) that participates in BRAF membrane docking after RAS-binding; CR2 (encoded by amino acids 360-375), containing the serine and threonine-rich hinge region, which bears important inhibitory phosphorylation sites, participating in the negative regulation of RAS binding and RAF activation. This CR2 provides a flexible linker that connects CR1 and CR3 and acts as a hinge; and CR3 (kinase domain, encoded by amino acids 457-717), makes up BRAF's enzymatic kinase domain: tyrosine kinase domain. The pathogenic variant V600E is located within this kinase domain. The smaller N-terminus lobe (residues 457-530) is primarily responsible for ATP binding while the larger C-terminus lobe (residues 535-717) binds substrate proteins. The active site is the cleft between the two lobes, and the catalytic Asp576 residue is located on the C-terminus lobe, facing the inside of this cleft [10,31].

The *BRAF* gene is expressed in most tissues, especially highest levels in neuronal tissues, testis, and haematopoetic cells. Mutations in *BRAF* gene can cause disease in two ways. First, in most cases, mutations can appear later in life: acquired i.e. somatic mutations occur randomly within a body cell, in this case, the tumor and cause cancer, as an oncogene. Here, in a few very rare cases, *BRAF* mutations can be inherited: germline mutations affect the sperm or eggs and therefore can be inherited from our parents, but further investigations are still needed for the confirmation of the hereditary manner of these mutations [32,33]. Second, mutations can be inherited and cause some rare genetic disorders such as cardiofaciocutaneous (CFC) syndrome, Noonan syndrome (NS), Costello syndrome, and LEOPARD syndrome (collectively known as RASopathies: a group of distinct but related congenital neurodevelopmental syndromes, which are characterized by cardiac defects, dysmorphic facial features, growth retardation and a variety of neiurological, cognitive, behavioral and/or motor coordination problems. RASopaties patients have an increased risk of cancer development) as well as birth defects [10]. Up to date, over 200 mutations in *BRAF* have been identified in cancer patients [31]. These mutations lead to the activation of the RAS-RAF-MEK-ERK pathway and based on the activity of the resulting BRAF protein, they have been divided into three classes: I-III, defined by their mechanisms of RAS dependency, dimerization status to induce the activation of the RAS-RAF-MEK-ERK pathway, and kinase activity [30,31] (Figure 9].

3. BRAF Inhibitors Against Cancer

The discovery of somatic mutation V600E (Val600Glu) in 2002 by Davies et al. [8] from melanoma patients was a major breakthrough in cancer research for targeted thera-

py. This mutation causes a change in conformation of BRAF (substitution of a non-polar valine,V, by a negative charged glutamic acid, E, at position 600 in the kinase domain of BRAF: V600E, blocks it in an activated state) resulting in increasing its kinase activity and leading to the activation of the RAS-RAF-MEK-ERK signal transduction pathway for promoting melanoma tumor growth. Somatic mutations in BRAF occur in numerous human cancers. One recurrent somatic mutation V600E, is frequently found in several tumor types, such as melanoma, colorectal cancer, lung cancer, thyroid cancer, etc. (Figure7). However, up to present, a germline mutation affecting codon 600 of BRAF and leading to the development of cancer has never been described. The timeline of the major U.S. Food and Drug Administration (FDA) approvals involving BRAF/MEK inhibitors is shown in Figure 10 [31]. Although the efficacy of BRAF inhibitors against the most common mutants has been shown but the emerging of resistance to therapy has been also reported. Combinatorial therapies including BRAF and MEK inhibitors as well as immunotherapy via immune checkpoint inhibitor, ICI, have been therefore explored and summarized in Table 1 [31]. For example, for BRAF V600E-mutant melanoma, the combination of Dabrafenib (BRAFi) plus Trametinib (MEKi) against Dabrafenib (BRAFi) alone as well as with Pembroliumb (anti-PD-1 mAb) or Atezolizumab (anti-PD-L1 mAb) has been applied (see case of the son (III-4) of the proband (II-2) and Table 1).

BRAF Inhibitors			
Drug	**Inhibitor Type**	**Approved to Treat**	**Date approved by the FDA**
Sorafenib (Nexlavar) Also: Sorafenib tosylate	1st-gen BRAFi	Advanced renal cell carcinoma Unresectable hepatocellular carcinoma Locally recurrent or metastatic, progressive, differentiated thyroid carcinoma (DTC) refractory to radioactive iodine treatment	20 December 2005
Vemurafenib (Zelboraf) Also: PLX4032	2nd-gen BRAFi	Unresectable or metastatic melanoma with BRAF V600E mutation Erdheim–Chester disease with BRAF V600 mutation	17 August 2011
Dabrafenib (Tafinlar) Also: GSK-2118436	2nd-gen BRAFi	Unresectable or metastatic melanoma with BRAF V600E mutation	29 May 2013
Encorafenib (Braftovi) Also: LGX818	2nd-gen BRAFi	(Not approved for use as a single agent)	27 June 2018
Combination Therapies			
Drug	**Inhibitor types**	**Approved to treat**	**Date approved by the FDA**
Dabrafenib + Trametinib (Tafinlar + Mekinist) Also: GSK-2118436 + GSK-1120212	Dabrafenib: BRAFi Trametinib: MEK1/2i	Unresectable or metastatic melanoma with BRAF V600E/K NSCLC with BRAF V600E mutation and involvement of the lymph node(s), following complete resection Locally advanced or metastatic anaplastic thyroid cancer with a BRAF V600E mutation Unresectable or metastatic solid tumors with BRAF V600E mutation and no satisfactory alternative treatment options Pediatric patients 1 year of age and older with low-grade glioma with a BRAF V600E mutation	9 January 2014
Vemurafenib + Cobimetinib (Zelboraf + Cotellic) Also: PLX4032 + GDC-0973	Vemurafenib: BRAFi Cobimetinib: MEK1/2i	Unresectable or metastatic melanoma with BRAF V600E/K	10 November 2015
Encorafenib + Binimetinib (Braftovi + Mektovi) Also: LGX818 + MEK162	Encorafenib: BRAFi Binimetinib: MEK1/2i	Unresectable or metastatic melanoma with BRAF V600E/K Metastatic NSCLC with BRAF V600E	27 June 2018
Encorafenib + Cetuximab (Braftovi + Erbitux) Also: LGX818 + Encorafenib	Encorafenib: BRAFi Cetuximab: monoclonal antibody, EGFR antagonist	Metastatic CRC with a BRAF V600E mutation (after prior therapy)	8 April 2020
Vemurafenib + Cobimetinib + Atezolizumab (Zelboraf + Cotellic + Tecentriq) Also: PLX4032 + GDC-0973 + RG7446	Vemurafenib: BRAFi Cobimetinib: MEK1/2i Atezolizumab: PD-L1 blocking antibody	Unresectable or metastatic melanoma with BRAF V600	30 July 2020

CRC: colorectal cancer; NSCLC: non-small-cell lung cancer.

Table 1: BRAF inhibitors and combination therapies currently FDA-approved to treat BRAF mutant cancers (Inspired from Ref. # 31).

Potential molecular link between AT and the RAS-RAF-MEK-ERK signal transduction pathway in inflammation, host defense, neurological manifestations, and cancer (melanoma)

Inflammation is the body's natural immune response to infection, injury, or toxins, aiming to remove harmful stimuli and initiate healing. The five cardinal signs of inflammation are pain, heat, redness, swelling, and loss of function. Inflammation is a generic response, and therefore is considered a mechanism of innate immunity, not adaptive immunity [34]. It involves immune cells (monocytes, basophils, eosinophils, neutrophils, lymphocytes), blood vessels, and molecular mediators (e.g. histamine, prostaglandins, leukotrienes, oxygen-and nitrogen-derived free radicals, and serotonin) released by immune cells. While acute inflammation is temporary and essential for repair, chronic inflammation is long-term (inflammation that lasts for months or years such as obesity, smoking, stress, etc. are some of the factors that promote chronic inflammation) and linked to DNA damages (due to the production of reactive oxygen species, ROS, and reactive nitrogen species, RNS) and a variety of diseases including diabetes, neurodegenerative disorders, cancer, arthritis, autoimmune diseases (in some diseases like arthritis and autoimmune diseases, the body's immune system triggers an anti-inflammatory response when there are no foreign invaders to fight off. The body responds as if normal tissues are infected or somehow abnormal), cardiovascular diseases, human immunodeficiency virus (HIV) and acquired immunodeficiency syndrome (AIDS), aging, depression, and delirium [35-44].

Hemostasis is the process to prevent and stop bleeding. It is the first stage of would healing. Coagulation, the changing of blood from a liquid to a gel, which forms the fibrin clots, is essential to hemostasis. Coagulation factors (CFs) such as thrombin, factor VIII and its partner Von Willebrand factor (VWF), tissue factor, as well as anti-coagulation factors are well-known class of proteins essential for hemostasis and preventing excessive blood clotting. One of the most important anti-CFs is AT, which inhibits most coagulant proteases of the coagulation cascade such as thrombin (Factor IIa), Factor Xa, Factor IXa, etc. [45]. Beyond hemostasis, angiogenesis is a physiologic process involving the growth of new blood vessels from pre-existing vessels and repair of vessel injury. Hemostasis occurs in combination with angiogenesis, which leads to new blood vessel formation. The new blood vessel formation are required for would closure and co-operate with the hemostatic system. Thus, there is a crossroad between hemostasis and angiogenesis. Note that under normal physiological conditions, angiogenesis is tightly regulated and is controlled by a balance of angiogenic stimulators and angiogenic inhibitors. Tumor growth is dependent upon angiogenesis and during tumor development in which a sustained production of angiogenic stimulatory factors is required along with a reduction in the quantity of angiogenic inhibitory factors tumor cells are produced [46].

Roles of the RAS-RAF-MEK-ERK signal transduction pathway in health and diseases such as inflammatory diseases, cardiovascular diseases, neurological and neurodevelopmental disorders, and cancers, etc. have been reported [10-13] (Figure 4), and there is a potential molecular link between AT, a pleiotropic protein (condition in which a gene product interacts with multiple proteins or catalyzes different reactions and affects multiple phenotypic traits) and this signal transduction pathway via epistasis: gene-gene interactions. Indeed, it is well documented that there are (a) bidirectional relation between inflammation and coagulation: inflammation leads to activation of coagulation but coagulation also significantly affects inflammatory activity. A down-regulation of anticoagulant pathways not only promotes thrombosis but also amplifies the inflammatory process. In contrast, activated coagulation process may affect specific cellular receptors on inflammatory cells and endothelial cells and thus modulate the inflammatory response [47-49]; (b) from a heterozygous Arg393His mutation in AT-Hanoi, AT, a pleiotropic protein, with multifaceted roles in intracellular functions outside of its anticoagulant function such as in inflammation (anti-inflammatory role), in host defense (antimicrobial and antiviral effects), anti-angiogenesis and tumorigenesis (via the cleaved, latent, and prelatent forms of AT [50-69]), and the "two-way association" between cancer and thrombosis have been demonstrated [1,4]; and (c) such a potential molecular link between AT and the RAS-RAF-MEK-ERK signal transduction pathway has been also clearly shown through the presence of melanoma (with BRAF V600E mutation) observed from the son (III-4) of the proband (II-2) (Figure1). Here, there are two events. First, there is implication of AT pathway in angiogenesis and tumorgenegis (Figure 11) and due to the Arg393His mutation in AT-Hanoi, it could not exert correctly its anti-angiogenesis and tumorigenesis. Second, there is the presence of BRAF V600E mutation that leads to the disregulation of the RAS-RAF-MEK-ERK pathway and resulting in the development of cancer such as melanoma. [10,26] (Figure 5). Given the highly variable thrombotic manifestations observed in AT deficiency ranging from asymptomatic individuals (case of I-2, deceased at age 97) to DVT, mesenteric thrombosis, encephalomalacia/gliosis and even cancer (kidney cancer: case of proband II-2), there are probably other yet undiscovered factors other than the genotype at the AT locus such as gene-gene interactions (epistasis) [70-72] as well as gene-environment interactions, such as surgery, pregnancy, hospitalization, diet, obesity, air pollution, etc. [1,4] should be emphasized as additional risk factors for VTE and severe thrombotic events. In this context, via epistasis, BRAF V600E mutation acted as a "modifier" gene (modifier genes are genes that influence, alter, or modulate the expression of other "target" genes, affecting the phenotype, severity of diseases, or genetic behavior with-

out directly causing the trait themselves. They can enhance, suppress, or change the function of other genes, often determining why individuals with the same disease-causing mutation show different symptoms) promoting the development of melanoma from the Arg393His mutation in AT-Hanoi through the RAS-RAF-MEK-ERK pathway as well as interconnection of cell signaling networks: cross-talk of signaling pathways in which one or more components of one signal transduction pathway affects another such as between RAS-RAF-MEK-ERK cascade and other pathways including PI3K/PTEN/AKT (this pathway promotes cell survival and growth) and Wnt (this pathway regulates cell fate and proliferation), rather than functioning alone [22,73]. Such an interaction of various signaling pathways is crucial in various "normal" physiological cellular processes in which a disregulation in each of these pathways is linked in the pathology of diverse human diseases including neurological manifestations such as AD, PD, ALS, RASopathies, and various types of cancers [10]. Note herein that BRAF-mutated melanomas are generally more aggressive than BRAF wild-type (WT) melanomas, and are more likely to metastasize to the brain [73]. When BRAF kinase is essentially activated due to a mutation (e.g. V600E), treatment with BRAF inhibitors results in blockade of downstream MAPK pathway. In cells with wild-type BRAF, treatment with BRAF inhibitors results in transactivation of CRAF and promotion of RAF dimerization, causing paradoxical activation of downstream MAPK pathway via the paradoxical activating role for BRAF inhibitors. In primary melanomas, mutations in other genes such as neuroblastoma RAS (*NRAS*), telomerase reverse transcriptase (*TERT*), tumor suppressor protein p53 (*TP53*), phosphatase and tensin homolog (*PTEN*), and cyclin-dependent kinase inhibitor 2A (CDKN2A) have been reported in which some of these mutations (in *BRAF*, *NRAS*, and *TERT*) were found both in benign lesions and in melanomas whereas those in *CDKN2A, TP53* and *PTEN* were only observed in invasive melanomas [73]. It was also reported that mutations in *BRAF* are independent of UV light (mutations by UV light are typically C→T nucleotide transitions at the 3' ends of pyrimidine dimers) [73]. So, *BRAF* mutations are not sufficient for melanoma development and progression as they are already also found in benign nevi. Melanoma development is, finally, an effect of other mutations in different genes, including *TP53, PTEN* and *CDKN2A*, as melanomas, genetically, are extremely heterogeneous, and they get many mutations from metastasis [73]. Here, via epistasis [70-72] and interaction of various signaling pathways including AT pathway, the presence of both mutations in *AT* and *BRAF* genes from the son (III-4) resulted favorably in the development of cancer, especially melanoma.

Perspectives

Cancer is a highly variable and complex disease that is characterized by uncontrolled growth and spread of abnormal cells potentially affecting almost any tissue in the body. Several factors, including genetics, lifestyle, and environmental factors such as polluted air, radiation, tobacco smoke, etc. contribute to the development of cancer. Most common cancers are polygenic (many genetic loci are involved, with each contributing a small portion to cancer risk). Cancer treatment has many options such as surgery, chemotherapy, radiation therapy, chimeric antigen receptor T-cell therapy (CAR T-cell therapy), immunotherapy, and targeted drug therapy. Depending on specific clinical factors such as cancer type, stage, and patient health status, one or a combination of these approaches is selected. Conventional cancer chemotherapeutic agents that indiscriminately affect all rapidly dividing cells are usually cytotoxic drugs [74]. CAR T-cell therapy is a personalized form of immunotherapy that trains your own immune cells to recognize and destroy cancer. It can be a powerful option for treating certain hard-to-treat blood cancers, especially when other treatments are no longer effective. However, CAR T-cell therapies can cause severe side effects such as cytokine release syndrome (CRS), immune effector cell-associated neurotoxicity syndrome (ICANS) as well as the risk of insertional oncogenesis associated with the use of retroviral vectors [75]. In contrast, targeted therapy focuses on molecular alterations specific to cancer cells, such as proteins or genes that drive cancer growth, thereby offering a highly precise approach leading to fewer side effects. Immunotherapy such as immune checkpoint inhibitor (ICI) that blocks immune check point protein (ICP) from binding with partner protein, is a distinct yet related class of targeted therapy aimed at enhancing host response to the tumor by enabling more effective cancer recognition and elimination. These two options of treatment have become the cornerstones of modern cancer treatment. Note that, for the treatment of melanoma, dacarbazine (DTIC: imidazole carboxamide, an alkylating chemotherapy causing methylation, modification and cross linking of DNA, thus inhibiting DNA, RNA, and protein synthesis. Common side effects include loss of appetite, nausea and vomiting [76]) was ineffective until targeted and ICI therapies were developed. The discovery of somatic mutation V600E (Val600Glu) in BRAF by Davies et al. [8] from melanoma patients was a major breakthrough in cancer research and mutated BRAF has become an important target for molecular therapy toward neurological manifestations, cardiovascular diseases, autism spectrum disorders as well as various types of cancers including melanoma [4,10-13,17,22,30,31,68, 73] (Figures 4,10) (Table1). However, epistasis [70-73,77-82], epigenetics (the study of how the environment and other factors can change the way that genes are expressed without changing the DNA sequence [73,83-87]), and the cross-talk of signaling pathways [22,73,88-93] as well as drug toxicities (with common side effects include fatigue, asthenia, ocular and dermatologic toxicities with visual disturbances, skin rashes, lesions in the

skin as well as in the liver, stomach, and intestines [94-98]), and drug resistance (this can be due to: overexpression of mutated BRAFV600E proteins which increase the frequency of BRAFV600E dimerization, while the inhibitors will act only on the BRAFV600E monomers, and results in the reactivation of the MAP/ERK pathway; frequency of BRAFV600E dimerization may also be affected indirectly by mutations in the *RAS* gene; splicing variants of BRAFV600E caused by mutations or epigenetic modifications making BRAF inhibitors ineffective; microheterogeneity of the tumors-some of the cells are wild-type in respect to BRAF, while some carry the BRAFV600E mutation. In BRAF wild-type cells, the expression of CRAF was shown to be higher and the BRAF inhibitors used such as vemurafenib stabilized BRAF-CRAF heterodimers, thus reactivating the MAPK pathway via the paradoxical activating role for BRAF inhibitors; loss of a functional PTEN protein involved in cell cycle regulation. This loss is observed in 10-35% of melanoma cases and results in the constitutive activation of the PI3K/AKT signal transduction pathway, leading to cell proliferation; complicated multifaceted role of melanocyte-inducing transcription factor, MITF, which is a regulator of the development and function of melanocytes. Both the overexpression and loss of MITF may contribute to reducing the therapeutic effect of BRAF inhibitors or MEK inhibitors in melanoma; role of the PI3K-AKT-mTOR pathway, which may also become activated and promote melanoma cell proliferation:- Mutations in the *PI3K-AKT* genes increase AKT signaling, which gives rise to antiapoptotic signaling and increases the expression of key proliferation genes, providing the cell with survival signals independent of BRAF or - With BRAF blocked, tumors cells can overexpress receptor tyrosine kinase, RTK, such as insulin-like growth factor 1 receptor, IGFR1, or platelet-derived growth factor receptor beta, PDGFRB, leading to permanent reactivation of PI3K/AKT signaling [73, 99-101]) make it challenging to achieve such an objective [73]. In any way, tumor cells have the ability to evolve new mechanisms for evasion of the immune system after its initial response such as secreting immunosuppressing factors, or altering their antigen expression, so limiting the ability of conventional T cells to recognize and kill them. Thus, as perspectives, the following approaches should be investigated:

- Exploration of the potential implication of AT (outside of its anticoagulant function) in the development of cancer, especially studying intermolecular interactions between AT and amyloid precursor protein (APP), BRAF, BRAFV600E, etc. [1,4];
- Combination therapy: combining two or more therapeutic strategies including the use of warfarin with anti-angiogenic, targeted, and immunogenic drugs would be beneficial for cancer patients [4]. As an example, combining engineered CAR T cells with ICI has been reported [4,102];
- Sonogenetics: a combination of genetic engineering and ultrasound (US) technology to activate sonosensitive mediators (SSMs) in specific cells to US stimulation to control biomolecular functions at the molecular level such as gene expression and modulation of cellular signaling pathways in order to alter the downstream effects thereby enabling precise control and remodeling outcomes [4,103-109]. Concerning SSMs, it is crucial to determine which ones show both structural and functional compatibility with sonogenetics responsiveness, and so used as sound-sensitive proteins (SSPs) toolboxes. The field of sonogenetics could have a lot of potential of applications for non-invasive therapy of neurological disorders, cardiovascular diseases, cancer, etc. (Figure 12). However, until now, like all emerging technologies, sonogenetics remains in the experimental stage and the translation to the clinic has been elusive because there are on going challenges that need to be addressed such as the ultrasound mechanism on cellular excitability.

For such issues, the construction of expression vectors via glycosylphosphatidylinositol (GPI) anchor performed according to [1,4] for studying intermolecular interactions would be useful (Figure 13).

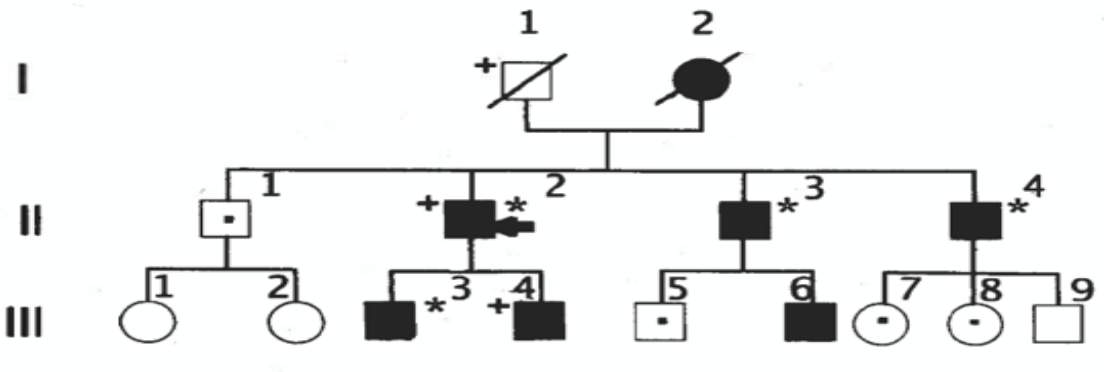

Figure1: Pedigree of the family. The proband (II-2) is indicated via an arrow. Thrombosis*; Cancer+; Presence of heterozygous Arg393His point mutation, solid symbols; Absence of heterozygous Arg393His point mutation, dotted symbols; Not investigated, open symbols; Deceased, dashed symbols. [Male ☐ Female **O**]

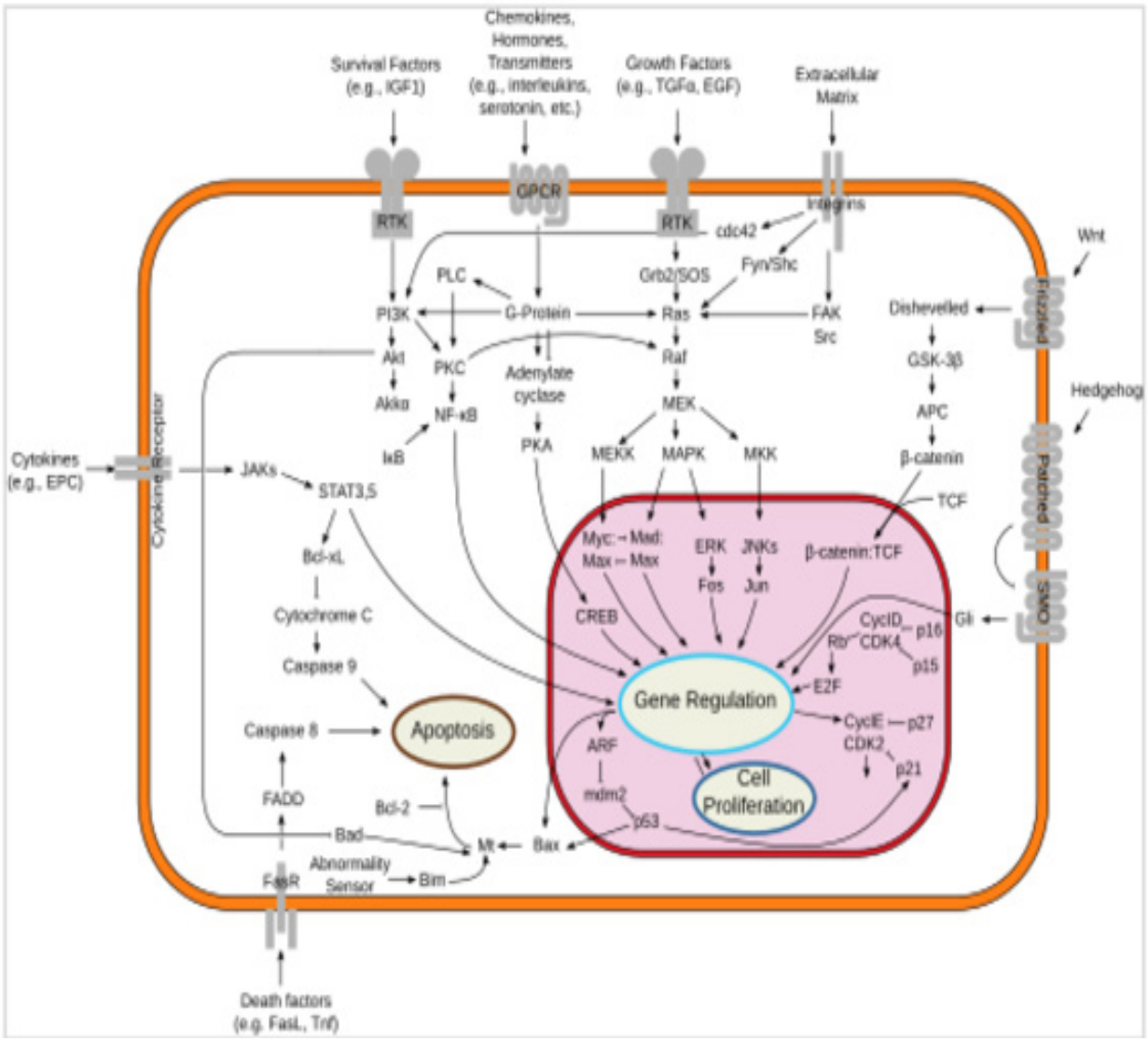

Figure2: Simplified representation of major signal transduction pathways in mammals and their interconnection networks. The RAS-RAF-MEK-ERK cascade is indicated in the center (see the signaling transduction pathway:...→ Grb2/SOS → Ras → Raf → MEK → MAPK →).

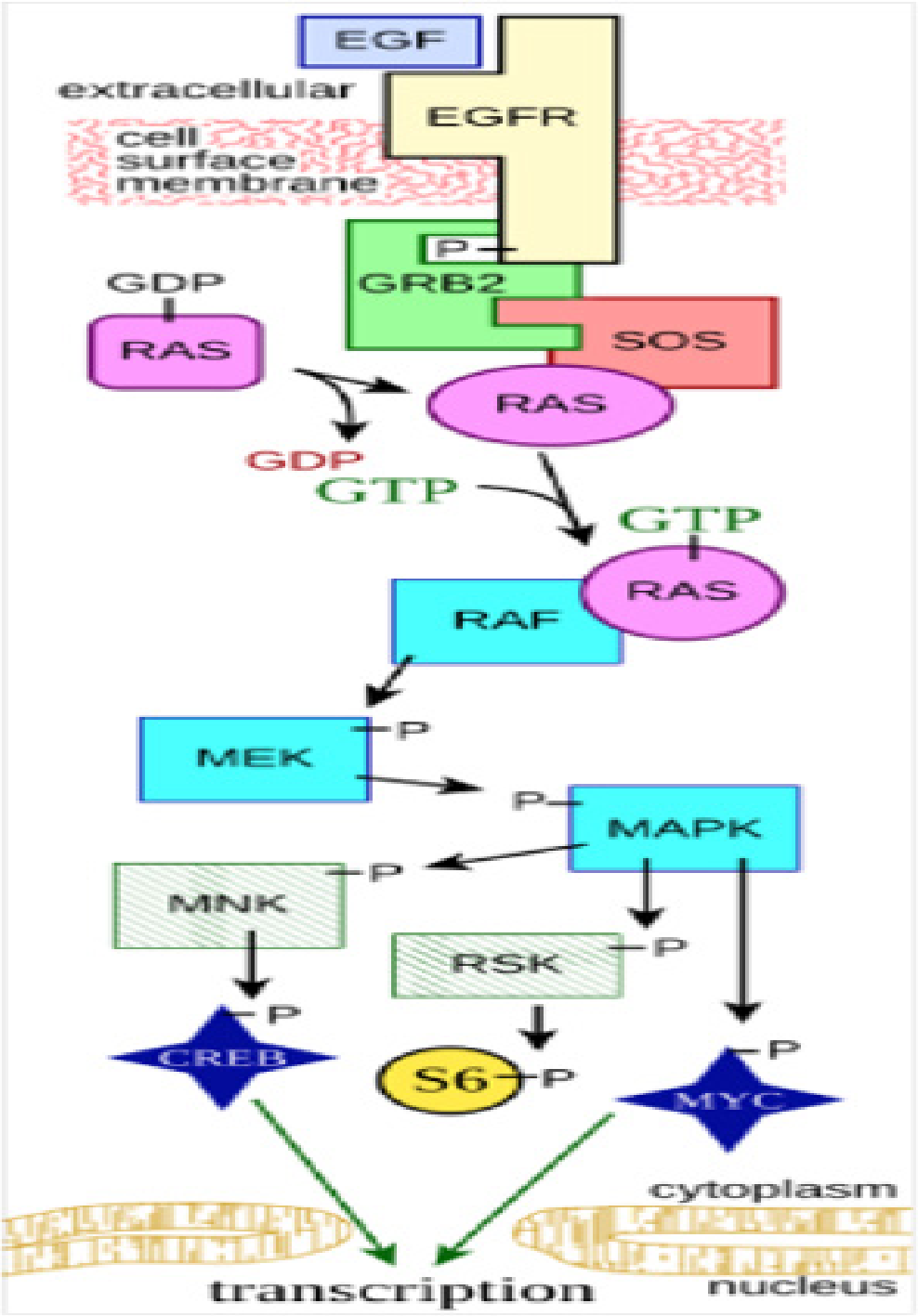

Figure3: Diagrammatic representation of the RAS-RAF-MEK-ERK signal transduction pathway. "P" represents phosphate, which communicates the signal. Top, epidermal growth factor (EGF) binds to the EGF receptor (EGFR) in the cell membrane, starting the cascade of signals. Further downstream, phosphate signal activates MAPK (also known as ERK). Bottom, signal enters the cell nucleus and causes transcription of DNA, which is then expressed as protein (see text for details in III- 1. RAS-RAF-MEK-ERK signal transduction pathway).

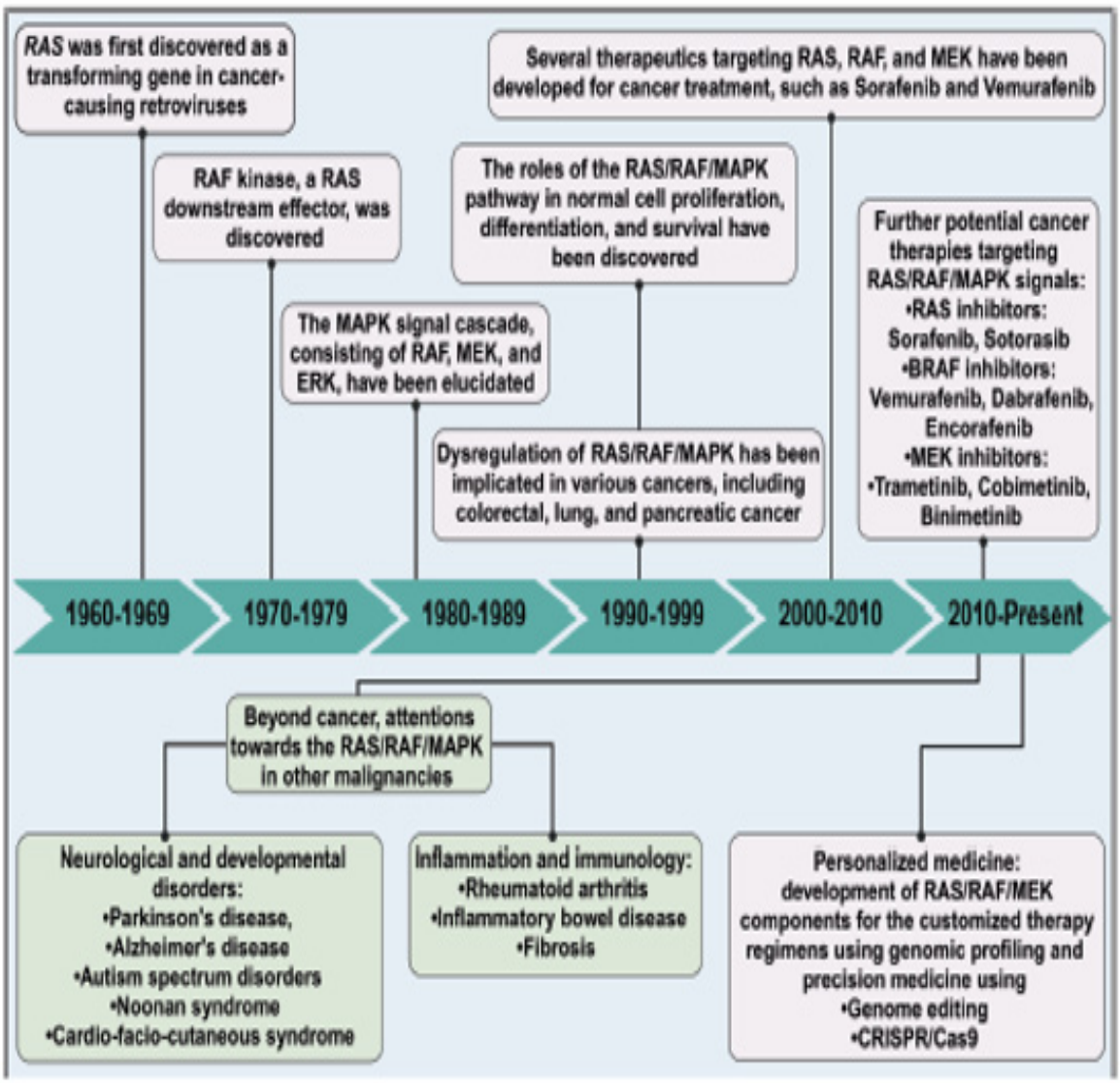

Figure4: Historical events of the discovery and development of the RAS-RAF-MEK-ERK (RAS-RAF-MAPK) pathway in health and diseases as well as anticancer drugs. The journey of the MAPK signal cascade commenced in 1960s with the groundbreaking discovery of the viral *RAS* gene. Subsequently, in 1992, the identification of RAF as both an upstream kinase activator of MEK and RAS effector marked significant milestones. These pivotal findings culminated in the comprehensive definition of the entire MAPK signaling pathway. Over time, the MAPK signal emerged as a critical component in the development of therapeutic strategies for combating cancer. Each of these major milestones in the RAS-RAF-MEK-ERK discovery is represented within its respective box. (Inspired from Ref. # 17).

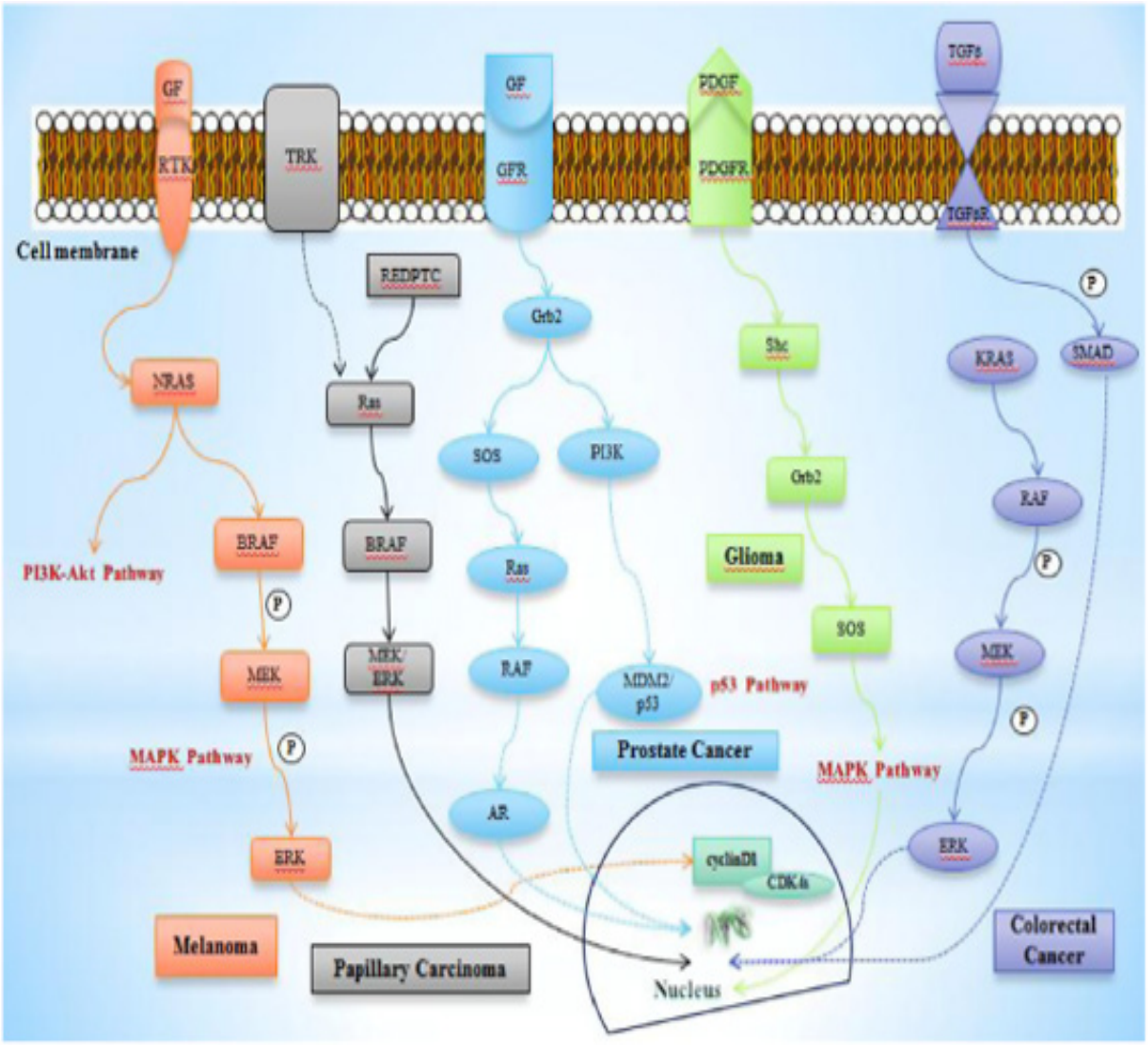

Figure5: Schematic diagram showing the RAS-RAF-MEK-ERK signaling pathway with different cancers. In melanoma, in red color, *RAS* and *BRAF* mutations active both effector pathways: RAF-MEK-ERK (*BRAF* gene) and PI3K-Akt signaling (GF: growth factor; RTK: tyrosine kinase receptor; PI3K: phosphatidylinositol 3-kinase; Akt: protein kinase B or PKB). In thyroid cancer (papillary carcinoma), in grey color, both *RAS* and *RAF* play a combined critical role in proliferation (TRK: tropomyosin receptor kinase; RET/PTC: rearranged during transfection/papillary thyroid carcinoma). Molecular cascade underlying prostate cancer (turquoise) underlies both MAPK and p53 pathways (GF: growth factor; GFR: growth factor receptor; SOS: son of sevenless; MDM2/p53: murine double minute 2 protein/tumor suppressor protein p53; AR: androgen receptor). The glioma, indicated in green color, involves PDGF, PDGFR, Sch, Grb2, SOS, RAS and MAPK (PDGF: platelet-derived growth factor; PDGFR: platelet-derived growth factor receptor; Shc: Src homologous and collagen protein; Grb2: growth factor receptor-bound protein 2). In colorectal cancer, indicated in blue color, both TGF-β and MAPK signaling pathways play a critical role (TGF-β: transforming growth factor beta; TGF-βR: transforming growth factor beta receptor; SMAD: suppressor of mother against decapentaplegic) (Inspired from Ref. # 10).

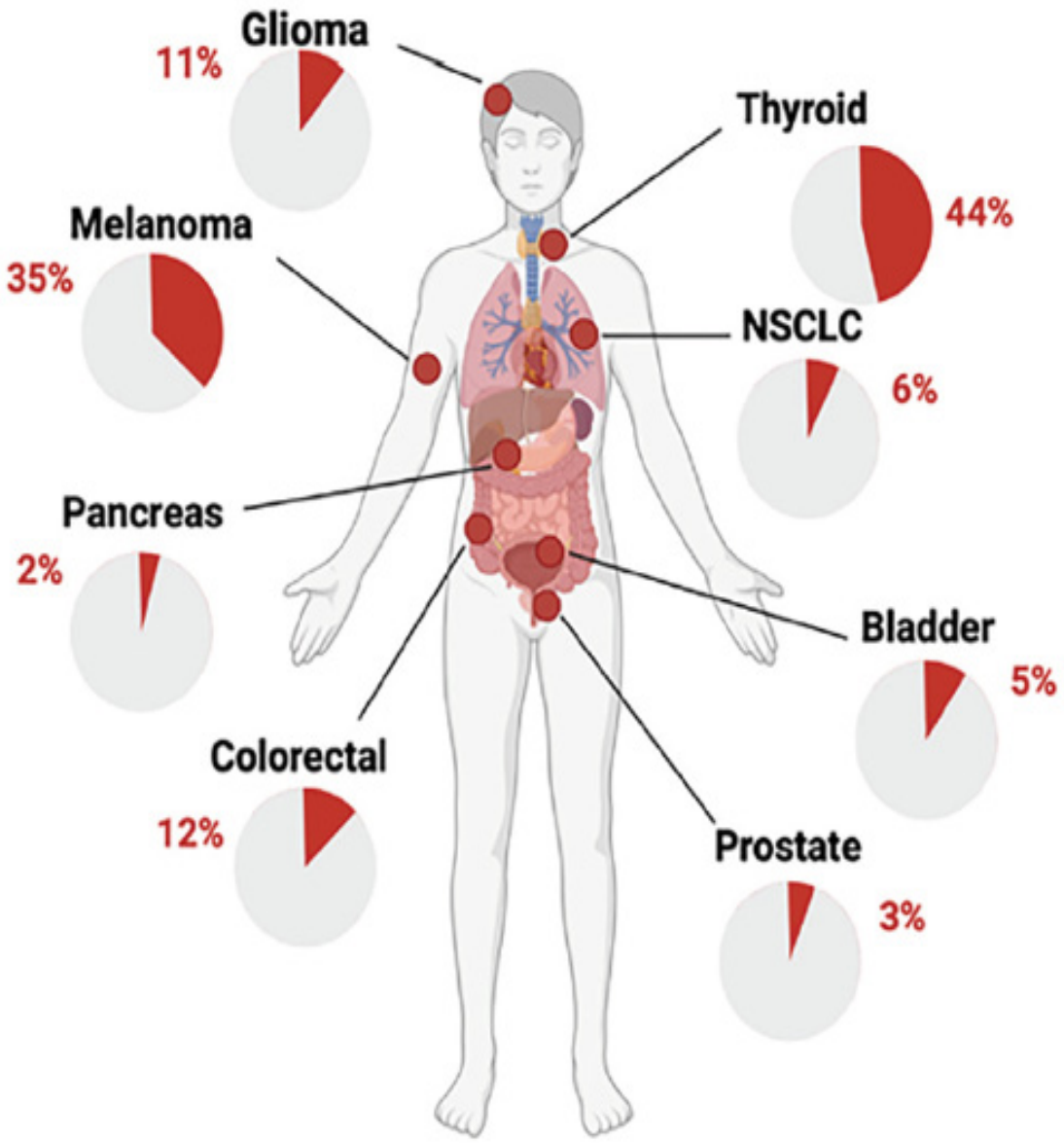

Figure6: BRAF alteration frequencies according to GENIE 15.1, among selected organ tumors (glioma, 11%; thyroid cancer, 44%; non-small cell lung cancer, NSCLC, 6%; bladder cancer, 5%; prostate cancer, 3%; colorectal cancer, 12%; pancreatic cancer, 2%; and melanoma, 35%) (Inspired from ref. # 30).

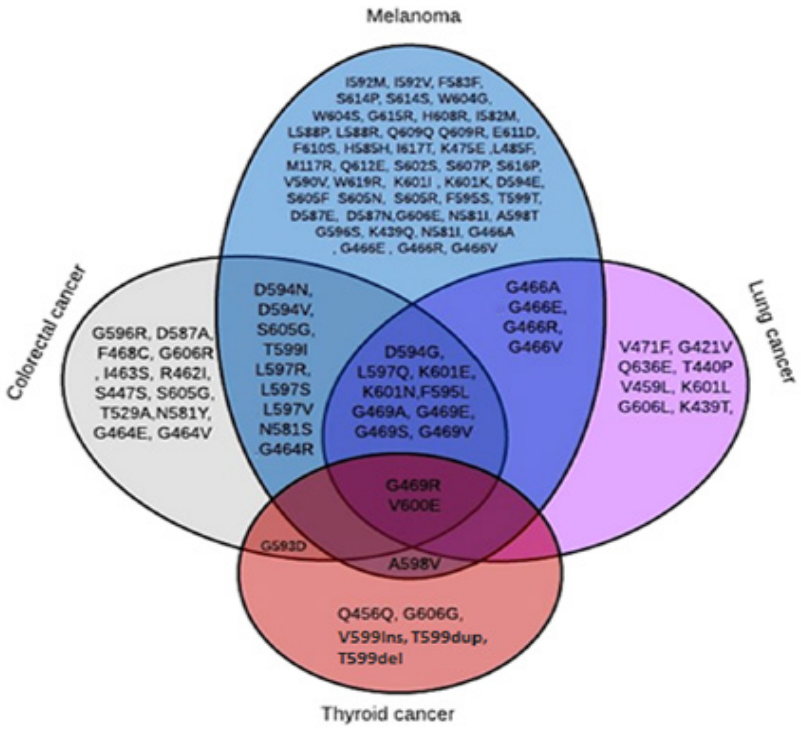

Figure7: Venn diagram to show BRAF mutations (amino acid variations), characterized in different cancer types. The highest number of mutations is seen in melanoma, indicated in blue color circle. The smaller number of mutations is observed in thyroid cancer, shown in red color circle. However, mutations observed in more than one type of cancer are shown in the overlapping region. For example, the G469R and V600E mutations are characterized in all 4 types of cancers, shown by the overlapping region. (Inspired from Ref. # 10).

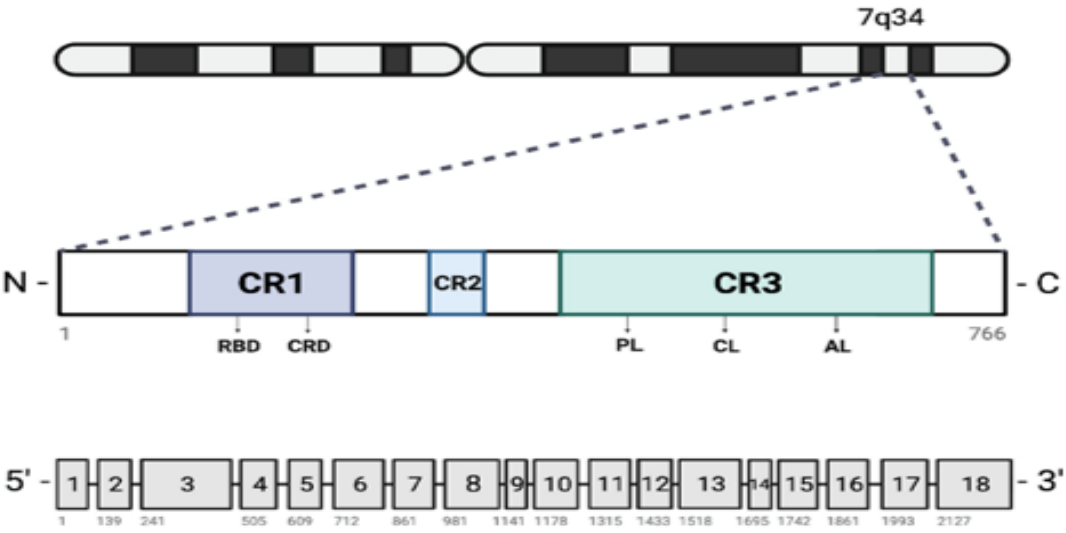

Figure8: Secondary structure of BRAF. [Top] Locus of *BRAF* gene on chromosome 7q34. [Middle] Secondary structure of the BRAF protein from amino acid 1 to 766, with conserved regions (CR) 1-3 and functional domains including the RAS-binding domain (RBD), cysteine-rich domain (CRD), phosphate binding loop (PL), catalytic loop (CL), and activation loop (AL). [Bottom] Representation of the 18 exons that make up BRAF, with the number of each exon's first base pair specified next to the gene (only counting the translated sequence, omitting untranslated region, UTR, and flanking sequences). (Inspired from Ref. # 31, see text for details in III-2. BRAF structure and mutation in cancer).

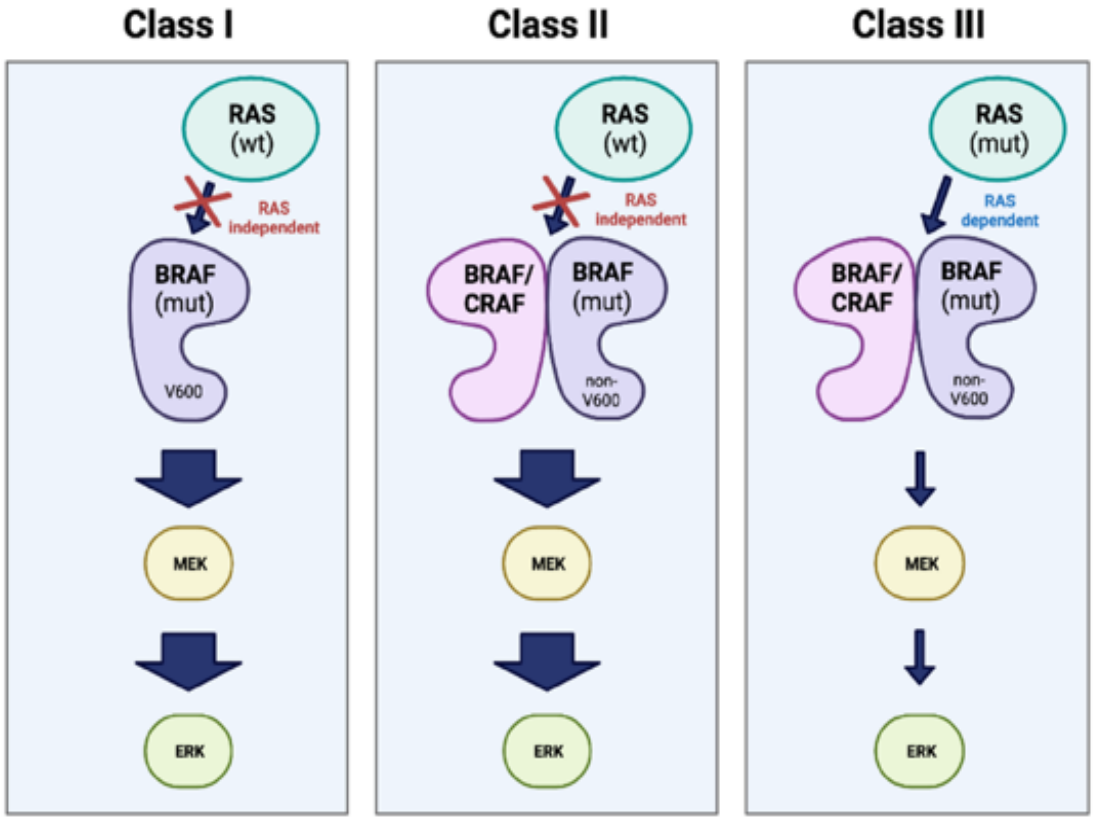

Figure9: Classes of BRAF mutation. Representation of the three major classes of oncogenic mutations in BRAF. Class I involves a RAS-independent, kinase-active BRAF monomer with a V600 mutation. Classes II and III are non-V600 mutations, and both act as dimers with either CRAF or a second BRAF. However, Class II is RAS-independent and results in high kinase activity, while Class III is dependent on mutant RAS and results in little to no kinase activity, represented in the figure by arrow thickness (Inspired from Ref. # 31).

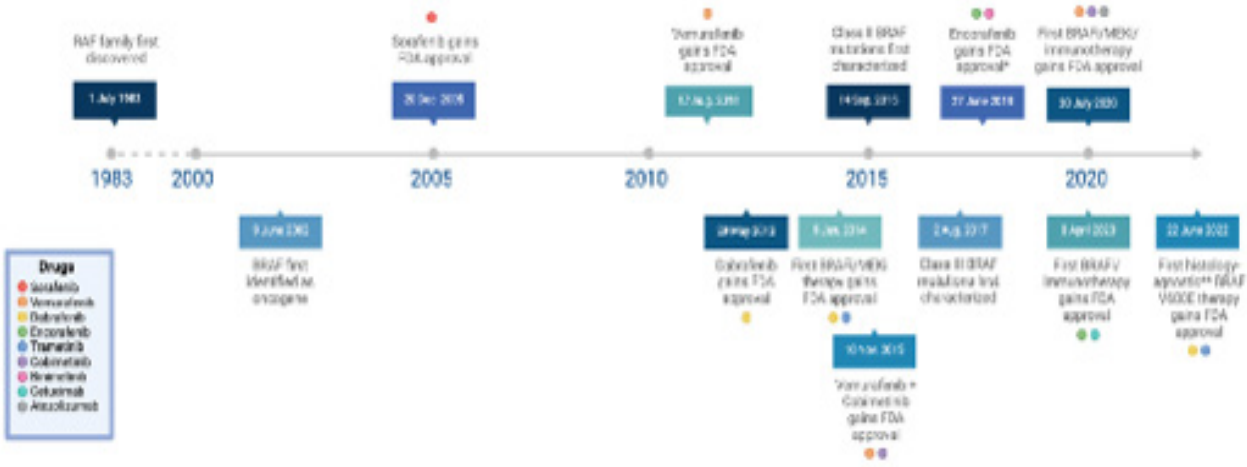

Figure10: Timeline of key advancements in the understanding of BRAF mutations breakthroughs in research, FDA approvals for novel treatment options, and the discovery of the three classes of BRAF mutations. (* Encorafenib not approved for monotherapeutic treatment, only in combination with other drugs) (** Histology-agnostic except for BRAF V600E-mutant colorectal cancer, CRC) (Inspired from Ref. # 31).

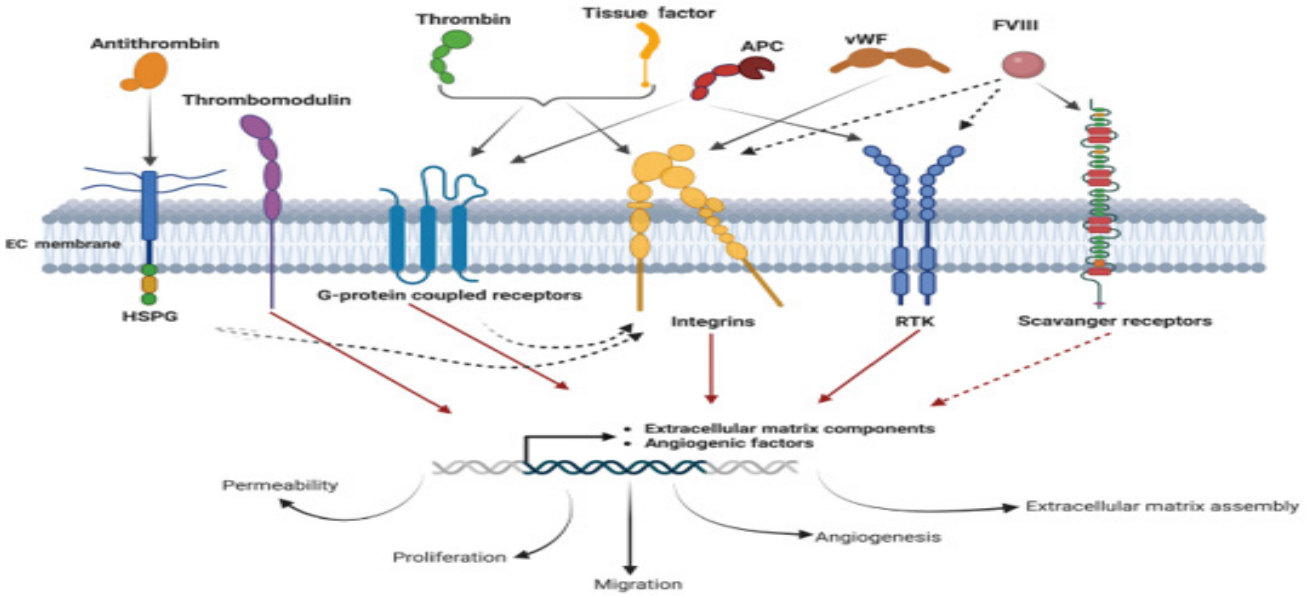

Figure11: Coagulation factors binding to receptors involved in endothelial cell functions. Antithrombin binds to heparan sulfate proteoglycans (HSPG) present on the surface of endothelial cells (EC) lining the vessel wall to exert its function in EC. HSPG have been described as interacting with integrins to regulate many EC functions via an interplay between the AT-HSPG complex and the extracellular matrix (ECM). Thrombomodulin seems to be implicated in angiogenesis regulation: thrombomodulin can induce the expression of genes involved in ECM and angiogenesis. Thrombin and tissue factor bind G-protein coupled receptors and integrins. Von Willebrand factor (VWF) binds integrin on EC and activated protein C (APC) binds G-protein coupled receptors and receptor tyrosine kinase (RTK). Factor VIII (FVIII) binds to known scavenger receptors, but its involvement with integrins or RTK in the regulation of EC functionalilty has never been fully explored. The activation of these receptors induces the expression of genes involved in ECM organization and angiogenesis modulating EC functions (Inspired from Ref. # 68).

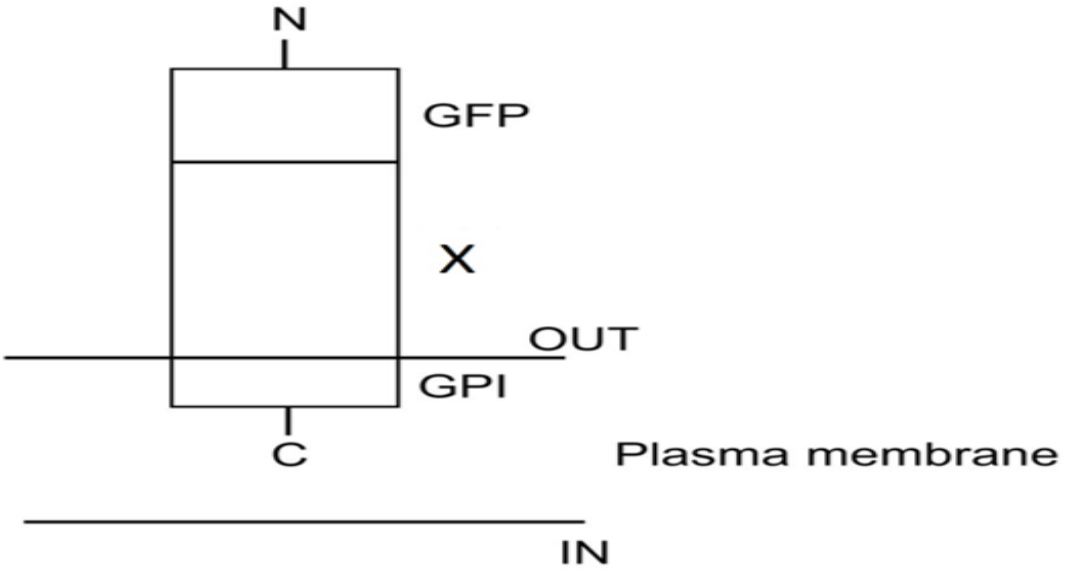

Figure12: Schematic presentation of the membrane topology of the expression vectors for human proteins via GPI anchor. The mammalian expression vector pcDNATM 3.1 (+) is used as backbone in which all genes of interest are inserted in the right frame into the pcDNATM 3.1 (+) vector. The construct comprising the sequence encoding the C-terminal of the glycosyl-phosphatidylinositol, GPI, anchor derived from the human folate receptor (FOLR1) protein; the entire coding sequence (CDS) of X (AT/ APP/BRAF/BRAFV600E/ICP/SSP, etc.) coupled with the CDS of the green fluorescence protein (GFP) gene (Inspired from Refs. # 1,4).

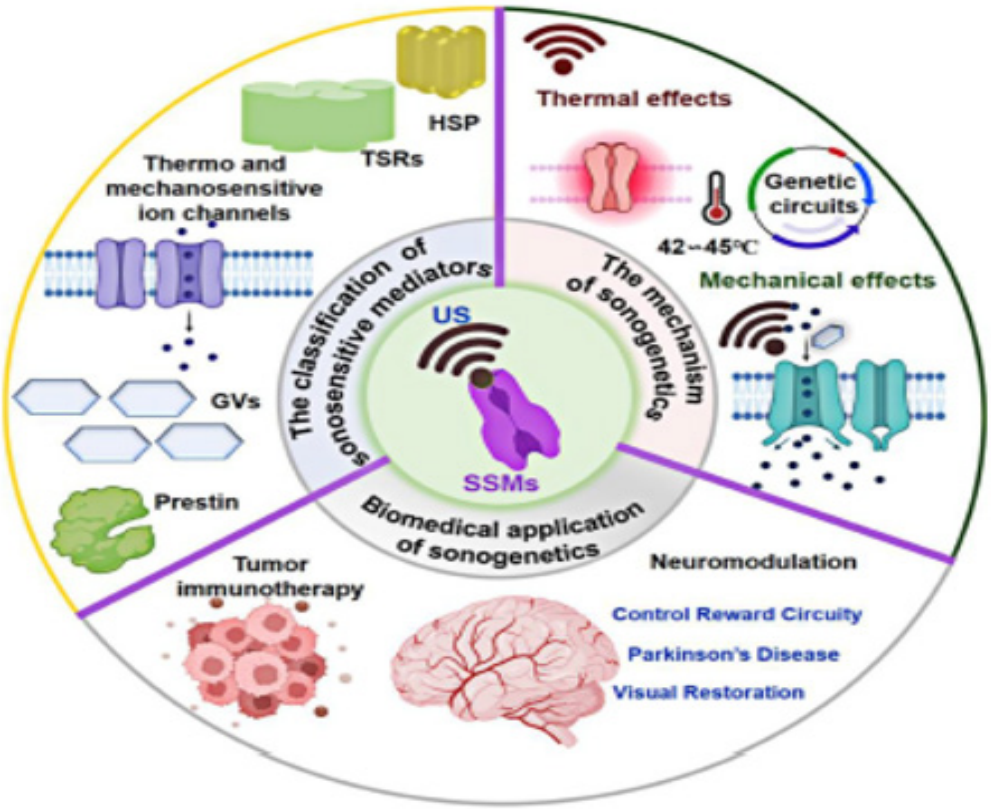

Figure13: Schematics design of the classification, generation, mechanism, and biomedical application of sonogenetics (Inspired from Ref. # 104) (US: ultrasound; SSMs: sonosensitive mediators; HSP: heat shock promoter; TSRs: temperature-sensitive repressors; GVs: gas vesicles; Prestin: an auditory-sensing protein found exclusively in outer cochlear hair cells. Prestin deficiency is associated with hearing loss in both humans and mice).

Conclusion

The present article demonstrates for the first time, a potential molecular link via epistasis and cross-talk of signaling pathways in which the mutation V600E (T1799A) of the *BRAF* gene acted as a "modifier" gene in the development of melanoma from an Arg393His antithrombin Hanoi deficiency's patient. This AT deficiency could be selected as a model of a rare hereditary disorder of blood clotting for the development of valuable drugs to modulating coagulation cascade and preventing therefore the consequences of blood clotting disorders that are in part responsible for cardiovascular diseases and neurological manifestations. Furthermore, this AT deficiency would be useful for studying the "bidirectional association" between cancer and thrombosis so that it would be useful for the development of cancer drugs, especially melanoma associated with BRAFV600E mutation.

List of abbreviations

AD: Alzheimer's disease
AIDS: acquired immunodeficiency syndrome
ALS: amyotrophic lateral sclerosis
APP: β-amyloid precursor protein
AT: antithrombin
BRAF: proto-oncogene B-RAF
CAR T-cell therapy: chimeric antigen receptor T-cell therapy
CDKN2A: cyclin-dependent kinase inhibitor 2A
CDS: entire coding sequence
CFC: cardiofaciocutaneous syndrome
CFs: coagulation factors
C- Fos gene: a proto-oncogene and transcription factor that plays an important role in many cellular functions and has been found to be overexpressed in a variety of cancers
C-myc: a family of regulator genes and proto-oncogenes, also sometimes referred to as MYC due to homology with the viral v-myc: an avian virus, myelocytomatosis
CRC: colorectal cancer
CREB: cAMP response element-binding protein
CRS: cytokine release syndrome
CVA: cerebrovascular accident
DTIC: imidazole carboxamide (dacarbazine)
DVT: deep venous thrombosis
EC: endothelial cell
EGF: epidermal growth factor
EGFR: epidermal growth factor receptor

eIF4E: eukaryotic initiation factor 4E
ERK: extracellular signal-regulated kinase
FDA: Food and Drug Administration
FGFR: fibroblast growth factor receptor
FOLR1: folate receptor1
GFP: green fluorescent protein
GPI: glycosylphosphatidylinositol
GRB2: growth factor receptor-bound protein 2
HDM2: homologue of the murine double minute 2 protein
HIV: human immunodeficiency virus
HRAS: Harvey rat sarcoma viral oncogene homolog
HSPG: heparan sulfate proteoglycan
ICANS: immune effector cell-associated neurotoxicity syndrome
ICI: immune checkpoint inhibitor
ICP: immune check point protein
IGFR1: insulin-like growth factor 1 receptor
INR: international normalized ratio
KRAS: Kirsten rat sarcoma viral oncogene homolog
mAb: monoclonal antibody, a homogenous collection of antibodies used to treat an illness. They are selected for their affinity to a target antigen
MAPK: mitogen-activated protein kinase
MEK: mitogen extracellular kinase
MI: myocardial infarction
MITF: melanocyte-inducing transcription factor
MNKs: MAPK interacting protein kinases
NS: Noonan syndrome
NRAS: neuroblastoma rat sarcoma viral oncogene homolog
NSCLC: non-small-cell lung cancer
PAD: peripheral artery disease
PD: Parkinson's disease
PD-1: programmed cell death1
PDGFR: platelet-derived growth factor receptor
PDGFRB: platelet-derived growth factor receptor beta
PD-L1: programmed death-ligand 1
PE: pulmonary embolism
PET: positron emission tomography
PTEN: phosphatase and tensin homolog
p53: tumor suppressor protein p53
RAF: acronym for rapidly accelerated fibrosarcoma
RAS: short for rat sarcoma virus
RASopathies: a group of distinct but related congenital neurodevelopmental syndromes (such as cardiofaciocutaneous, CFC, syndrome, Noonan syndrome, NS, Costello syndrome, and LEOPARD syndrome), which are characterized by cardiac defects, dysmorphic facial features, growth retardation and a variety of neiurological, cognitive, behavioral and/or motor coordination problems. RASopaties patients have an increased risk of cancer development
RNS: reactive nitrogen species
ROS: reactive oxygen species
RSK: ribosomal protein S6 kinase
SERPINC1: serpin peptidase inhibitor, clade C, member 1
SOS: son of sevenless
SSMs: sonosensitive mediators
SSPs: sound-sensitive proteins
TERT: telomerase reverse transcriptase
Trk: tropomyosin receptor kinase
US: ultrasound
VTE: venous thromboembolism
VWF: Von Willebrand factor
WHO: World Health Organization

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