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Review Article

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Regulation of cell proliferation and tumor suppressor roles of microRNA 329-3p of the MAP kinase pathway in cervical squamous carcinoma

Elfansu[1], Faria Rashid[2], K. Abirami[3], Sivakumar Krishnamoorthy[4], Kshitija Aherkar[1], R. Mythreyi[1],Uthamalingam Murali[5], Kanthesh M. Basalingappa[1]*, Selvaraj Jagannathan[6], E. Boojhana[7], M. Maghimaa[7]*

[1]Division of Molecular Biology, School of Life Sciences, JSS Academy of Higher Education and Research, Mysuru-570015, Karnataka, India, [2]Tenby International School Ipoh, Malaysia, [3]Department of Microbiology, Vivekanandha College of Arts and Science for Women, Tiruchengode, Namakkal- 637205, Tamil Nadu, India, [4]Agricultural Microbiology, Institute of Horticulture, Horticulture Research Station, Tamil Nadu Agriculture University, Pechiparai, Kanyakumari-629161, Tamil Nadu, India, [5]Department of Surgery, Manipal University College Malaysia, Malaysia, [6]Department of Microbiology, Pasteur Institute of India, Coonoor, The Nilgiris-643103, Tamil Nadu, India, [7]Department of Microbiology, Muthayammal College of Arts and Science, Rasipuram, Namakkal-637408, Tamil Nadu, India

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***Corresponding authors:**
Kanthesh M. Basalingappa
E-mail: kantheshmb@jssuni.edu.in
M. Maghimaa
E-mail: maghimaamm@gmail.com

ABSTRACT

Cervical squamous cell carcinoma is observed as the second major cause of mortality worldwide. A highly conserved mitogen-activated protein kinase (MAPK) signaling pathway occurs in a wide range of cellular processes which includes differentiation, proliferation, migration, senescence, and apoptosis. MAPK pathway can be activated by various extracellular signals, capable of generating responses as per the cell type. Thus, alteration of the EGFR receptor in this particular pathway leads to the condition of cancer due to abnormal activation of receptor tyrosine kinases. The characteristic features of microRNA (miRNA) which are endogenous, single-stranded, small non-coding RNA for their role in RNA silencing and post-transitional regulation of gene expression have been studied over the years. The miRNA functions by base pairing with the complementary sequences within the mRNA molecule. One such miRNA, miR-329-3p has a critical tumor suppressor role in the MAPK pathway, however, is least understood. Therefore, miRNA could be considered as a potential biomarker for diagnosis, prognosis, and therapeutic purposes and brought out to its fullest use to mankind.

KEYWORDS: Cervical cancer, Squamous cell carcinoma, Biomarker, Proliferation, Metastasis, Apoptosis

INTRODUCTION

Cancer is a wide range of diseases characterized by the uncontrollable division of cells with the ability to infiltrate and destroy normal human tissue (Wani, 2023). When cancer begins, normal cells in that area of the body start to proliferate in an uncontrolled manner as a consequence of a change in one gene of one cell or a few cells which eventually begins to grow and multiply in an uncontrolled manner. Primary cancer refers to the location in the body where cancer first develops. The original site in the cells may separate and disperse to various body regions (Chanu & Singh, 2022). Subsequently, these cells may develop into new cancers. These are referred to as metastases or secondary cancers. Any aberrant cell proliferation, whether benign or malignant, is referred to as a tumor (Hanahan & Weinberg, 2000; Chanu & Singh, 2022). A benign tumor stays in its original place and does not invade nearby tissue; however, a malignant tumor can invade the normal tissue around it and to travel throughout the body through the lymphatic or circulatory systems (Hanahan & Weinberg, 2000).

Being a genetic disease, the cancer can arise from factors categorized mainly as hereditary or environmental. However, the majority of the occurrences are due to mutations of the gene that arise from a plethora of environmental factors (Anand *et al.*, 2008). Some of these include tobacco (25%-30%), diet and obesity (30%-35%), infections (15%-20%), radiation (both ionizing and non-ionizing up to 10%), age, diet, obesity, stress, lack of physical activity and pollution (Hanahan & Weinberg, 2000; Kolonel *et al.*, 2004; Anand *et al.*, 2008).

Over 200 different types of cancer are known to exist with the cases increasing at an alarming rate over time (Mohemed *et al.*, 2023). As per the origin of the site, cancer can be classified as thyroid cancer, breast cancer, lung cancer, cervical cancer, colon cancer, thyroid cancer, etc.

CERVICAL CANCER

The uncontrolled growth and proliferation of the cells at the cervixis termed "cervical" cancer. The cervix forms the lowest portion of the uterus that joins the vagina and has two major parts - the lower part projects into the vagina and is termed as vaginal part, while the upper part is called the supra vaginal part. It is conical in shape. The cervix connects the body of the uterus with the vagina or the birth canal at the external osteum (Sellors & Sankaranarayanan, 2003). The part of the cervix close to the uterus is named as endocervix which is lined by squamous cells while the part next to the vagina is called as exocervix which is lined by glandular cells (Sellors & Sankaranarayanan, 2003). The zone merging the two kinds of cells is called the squamous columnar junction (SCJ) or the transformation zone that forms the major site of infection and occurrence of cancer (Sellors & Sankaranarayanan, 2003). Cervical cancer is divided into three subgroups based on histology: squamous cell carcinoma, adenocarcinoma, and adenosquamous carcinoma (Sellors & Sankaranarayanan, 2003; Li *et al.*, 2017). Squamous cell carcinoma is the most frequent of these subtypes, accounting for 85% of all occurrences (Li *et al.*, 2017).

According to the Cervical Cancer Statistics, cancer of the cervix uteri is found to be the fourth most common cancer affecting women on a global scale and the second most common cancer in developing regions (Zheng *et al.*, 2015; Li *et al.*, 2017). The estimated global incidence is about 5,30,000 women develop cervical cancer each year and nearly 2,70,000 women deaths are recorded annually (Sellors & Sankaranarayanan, 2003; Zheng *et al.*, 2015; Li *et al.*, 2017). India has a population of 432.2 million women and every year around 122,844 women are diagnosed with cervical cancer and 67,477 die from the same (Sellors & Sankaranarayanan, 2003; Zheng *et al.*, 2015; Li *et al.*, 2017). Most of the cases, that is, around 85% are found to be present in advanced and late stages, and more than half that is from 63% to 89% have regional disease at the time of presentation. Therefore, preventive vaccination and organized screening programs are critical for the identification of cervical cancer. Thus, an understanding of the molecular mechanisms of cervical cancer development and the identification of novel biomarkers are essential for the early detection and treatment of cervical cancer.

Cervical cancer is mainly due to a persistent infection with high-risk types of the human papilloma virus (hrHPV) (Bowden *et al.*, 2023). The human papilloma virus (HPV) contributes to more than 90% of cases while other causes include smoking, birth control pills, sexual intercourse at an early age, a greater number of sexual partners, a weak immune system, etc (Zheng *et al.*, 2015; Bowden *et al.*, 2023). Genetic instability due to the presence of a transforming infection can ultimately result in the accumulation of specific genetic and epigenetic changes in the host-cell genome. This, in turn, drives progression to a malignant phenotype. The oncogenic cell transformation is found to occur in a discrete cell population at the SCJ (Zheng *et al.*, 2015; Bowden *et al.*, 2023). The uncontrolled growth of cells leads to lumpy regions in the cervix that can spread to distant sites via metastasis. In the majority of cases, the mortality is due to this metastatic behavior of the cancer cells that invade other organs of the body from the primary location of the invasion (Zheng *et al.*, 2015; Bowden *et al.*, 2023). Thus, early detection and diagnosis as done by processes including biopsy proves to be helpful. Generally, to estimate the spread of tumors medical imaging is done. Screening is typically by a PAP smear test that helps to identify the precancerous change occurring in the body. Thus, on early detection, the occurrence of cancer can be prevented (Bhatla *et al.*, 2018; Basoya & Anjankar, 2022; Bowden *et al.*, 2023).

There are various molecular mechanisms including pathways such as PCP/WNT, BETA catenin, RAS/RAF, JNK, AKT, PCT, etc. that play a vital role in the normal functioning of the body. Among these, the oncogenic virus infection that is caused by a classical pathway is the MAP kinase pathway. Any pathway has basically few important criteria which include:

- Signaling molecules - They are generally hormones that include epidermal growth factor hormone (EGFH) and plasma-derived growth factor (PDGF).
- Receptor - They are usually transmembrane proteins like Epidermal growth factor receptors.
- Core signaling molecule and some messengers that involve kinases.

MICRO RNA

The micro RNAs (miRNAs) are single-stranded, small non-coding RNA molecules that consist of a short nucleotide single-stranded region. These are found in all plants, animals, and some viruses and are involved in the RNA silencing and post-transitional regulation of gene expression (Ying *et al.*, 2008). They are known to play important roles in a wide range of physiological and pathological processes that occur within the body including important regulatory roles in cellular proliferation, apoptosis, angiogenesis, invasion, and migration (Ying *et al.*, 2008). It affects the activities of other genes involved in protein synthesis by being transcribed from DNA as opposed to being translated into protein (Ying *et al.*, 2008).

miRNA, which includes the lin-4 and let-7 genes, were first discovered in *C. elegans* as 18-23 nucleotide long RNA molecules that are complementary to the 3′ untranslated regions of their target transcripts and were later discovered to exist in a variety of animals, including worms, flies, and humans (Ying *et al.*, 2008; Bhatla *et al.*, 2018). Most miRNAs are produced via transcription of DNA sequences into fundamental miRNAs, precursor miRNAs, and then mature miRNAs. The majority of the time, miRNAs interact with the target mRNAs' 3′ untranslated region to cause mRNA degradation and translational suppression (Allmang *et al.*, 1999; Ying *et al.*, 2008).The study over the years has shown that miRNAs are organized into highly conserved

families with common seed regions of occurrence of about 6-8 nucleotides which determine the target specificity (Allmang *et al.*, 1999). The miRNA functions via the base pairing with the complementary sequences inside the mRNA molecule1 (Allmang *et al.*, 1999; Bartel, 2009). The human genomes comprise of about 1000 miRNAs and are found to affect the target of about 60% of the genes of humans and other mammals (Abbott *et al.*, 2005; Bartel, 2009).

miRNA binds to target mRNA to block the creation of proteins through one of two different processes. Primary miRNA which joins the effect or complex RNA-induced silencing complex (RISC), is cleaved twice to produce mature miRNA (Abbott *et al.*, 2005; Betel *et al.*, 2008). By base-pairing with the target mRNA to inhibit its production, the miRNA acts as a guide (O'Brien *et al.*, 2018).

The importance of miRNA in human carcinogenesis is becoming increasingly recognized, and the potential role of miRNA in cervical cancer development is being developed and demonstrated in only a few studies. The recent work on miRNA functioning has revealed that miRNA could negatively modulate its matched target genes by binding the 3' untranslated regions (3'UTRs) of mRNA, leading to mRNA degradation and thus translation inhibition (Betel *et al.*, 2008; O'Brien *et al.*, 2018). The silencing of the RNA can be performed by; a) Cleavage of mRNA strand into pieces. b) Destabilization of the mRNA through shortening of its poly (A) tail and c). The translational of less efficient mRNA into proteins by ribosomes (Betel *et al.*, 2008; O'Brien *et al.*, 2018).

Each miRNA has numerous gene targets, and a particular gene can be targeted by multiple miRNAs providing a combinatorial effect on the gene regulation (Vasudevan, 2012). Human miRNA is located at fragile sites on the chromosomal regions that are affected by cancer.

The miRNAs can be detected depending on their genomic location and are found in both coding and non-coding genes as they consist of introns and exons. They share the expression pattern and regulation along with the host cells (Bentwich *et al.*, 2005; Vasudevan, 2012). Thus, they can regulate many targeted gene expressions, which include proliferation, invasion, apoptosis, migration, and differentiation (Griffiths-Jones, 2004; Bentwich *et al.*, 2005; Vasudevan, 201). Many studies have provided evidence that a variety of miRNAs are involved with the initiation and progression of human malignancies. Several studies have shown altered miRNA expression patterns in cervical cancer cell lines and cervical cancer tissues (Lewis *et al.*, 2005). So far, only limited data on genome-wide miRNA expression patterns in CIN lesions is available (Lewis *et al.*, 2003, 2005). Thus, the role of microRNA in inhibition of proliferative cells in the MAPK pathway and hence the downregulation of cancer cells in the cervical region plays a crucial role. Over the last decade, an increasing number of studies have found that miRNAs play an important role in carcinogenesis and progression. MiRNAs can behave as oncogenes by negatively influencing tumor-suppressor genes, whereas lowly expressed miRNAs can function as tumor suppressors by directly targeting oncogenes (Lewis *et al.*, 2003; Li *et al.*, 2017). As a result, identifying novel miRNAs to act as therapeutic targets in human cancer may be advantageous.

Many miRNAs including microRNA 29a, microRNA 362, microRNA 329 3p, microRNA 133a, microRNA 139 3p, microRNA 338 3p, etc. are known to play crucial roles as per their specific mechanism of action. The validation and approval are still under study with the recorded data. One such microRNA, miRNA 329 3p is thought to serve as a predictive marker since its expression in cancer cells, was found to be at a lower expression level as known for lymph nodes and metastasis (Rajewsky, 2006; Liu *et al.*, 2007; MacFarlane & Murphy, 2010; Li *et al.*, 2017). The upregulation of the same, on the other hand, is related to suppression of the cell proliferation, migration, and invasion in the cancer cells (Cullen, 2004; John *et al.*, 2004; Rajewsky & Socci, 2004; Krek *et al.*, 2005; Liu *et al.*, 2007; Zhao & Srivastava, 2007; Fabian *et al.*, 2010). Recent evidence suggests that miR-329-3p not only suppresses tumors but also plays a role in tumor cell proliferation and spread in various cancers (Zeng & Cullen, 2003; Ambros, 2004; Cullen, 2004; Aravin & Tuschl, 2005).

Thus, considering the silencing of the MAPK1 via the direct target gene miRNA 329 3p leads to the process of RNA interference (Perron & Provost, 2008; Bartel, 2014). Consequently, it is suggested that the miR-329-3p has a critical tumor suppressive role wherein it directly targets the MAP Kinase pathway in cervical cancer.

THE ROLE OF THE MAP KINASE PATHWAY

The MAP kinase pathway is generally a signal transduction pathway that regulates the communication between the extracellular signals and the cell machinery. This controls the functional processes such as cell growth, proliferation, apoptosis, differentiation, migration, and invasion (Kyriakis *et al.*, 1994). Figure 1 displays the total pathway representation of MAP Kinase. The pathway originated from one of the first members of the protein family and is hence called the mitogen-activated protein (MAP) kinases pathway. This pathway is regulated in three levels of phosphorylation cascades. The induction of the pathway depends on the kinases which include the receptor tyrosine kinases (RTKs) that can be activated by the attachment of the ligand (Sun *et al.*, 2015). The RTKs are the surface receptors for many of the growth factors, hormones, cytokines, and extracellular signaling molecules. They comprise the subfamily- Epidermal growth factor receptors (EGFR), Fibroblast growth receptor (FGFR), insulin and insulin-like growth factor receptors (IR and IGFR), platelet-derived growth factor receptors (PDGFR), Hepatocyte growth factor receptors (HGFRs), vascular endothelial growth receptors (VEGR), etc. (Dérijard *et al.*, 1994; Sun *et al.*, 2015). The activation of the RTKs provides further induction of the phosphorylation process. This involves downstream kinases leading to the phosphorylation of the targeted protein.

The transforming growth factor alpha (TGF alpha) is a protein coded by the TGFA gene. It is a mitogen polypeptide (Burotto

et al., 2014; De Luca *et al.*, 2012). As a member of the epidermal growth factor family, the protein becomes activated by binding to the receptors, which undergo protein kinase activity for cellular signaling (Robinson & Cobb, 1997; Wan *et al.*, 2004; De Luca *et al.*, 2012; Burotto *et al.*, 2014). It serves as a ligand for EGFR thereby activating the signaling pathway. It may act in two ways: the bound ligand or the ligand of the soluble receptor tyrosine kinases on action with the receptor of EGFR (Epidermal growth factor receptor) undergoes phosphorylation and hence results in the attachment with the SOS (sons of seven less) which are guanine encoding exchange factors along with the Grb2 (Growth factor receptor bound protein 2) (Robinson & Cobb, 1997). Table 1 demonstrates the mammalian MAP Kinase pathway.

The factor brings SOS and the protein adapter Grb2 is in close proximity to the plasma membrane. The smaller GTPase and RAS results in the nucleotide exchange of GDP to GTP (i.e., Guanine diphosphate to guanine triphosphate) (Robinson & Cobb, 1997; Wan *et al.*, 2004; De Luca *et al.*, 2012). This substitution reaction leads to the activation of serine/tyrosine kinase RAF/MAP kinase kinase kinase (MAPKKK) that occurs as a result of the internal phosphorylation and the utilization of the ATP leading to further activation of the MAP kinase (MAPKK) and finally, the MAP kinase in its active form (Boulton *et al.*, 1991; Robinson & Cobb, 1997; Scaltriti & Baselga, 2006). Thus, a cascade of reactions occurs which undergoes phosphorylation including the RAF phosphorylation of MEK½ (methyl ethyl ketone) which in turn phosphorylates and activates Erk½ (Yarden & Ullrich, 1988; Kaplan *et al.*, 1991).

The MAP Kinase pathway is involved in tumor suppression, increased expression of phosphorylation of several transcription factors such as the p53, activating transcription factor 2 (ATF2), E1K1, protein kinases, MAPK activated kinases 2 (MK2)/(MAPK2), nitrogen and stress-activated protein kinases1 MAP kinase interacting serine/threonine kinase (Zhang & Liu, 2002).

TARGETING THE RAS/RAF PATHWAY IN CERVICAL CANCER

RAS proteins are proto-oncogenes that are encoded by three distinct genes that are widely expressed: HRAS, KRAS, and NRAS. These proteins are GTPases, which act as molecular switches, controlling pathways involved in cell growth and

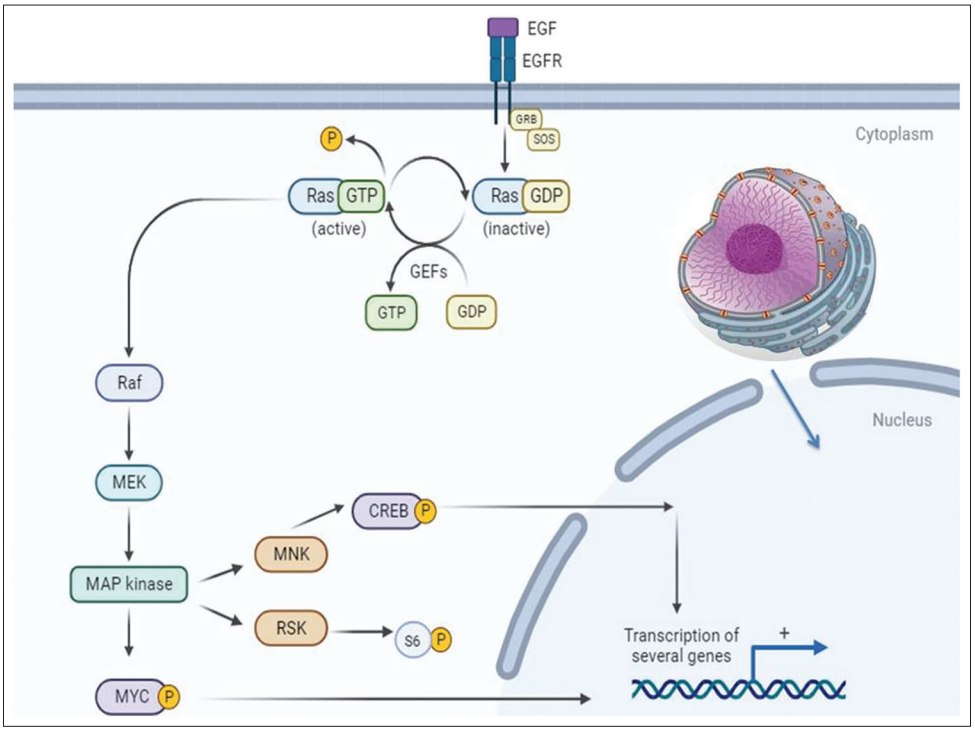

Figure 1: Total pathway representation of MAP Kinase pathway

Table 1: Map Kinase Pathway (Mammalian)

S. No.	Map Kinase	Other Names	Comments	P Site Motif	References
1	ERK1	p44 MAPK	>80% identical to ERK2 Abundant and ubiquitous	TEY	45
2	ERK2	p42 MAPK	Abundant and ubiquitous	TETY	42
3	MRK	MAK related kinase	Expressed in embryonic myocardium.	TDY	60
4	JNK1	SAPK	Multiple spliced forms	TPY	42,45
5	JNK2	SAPKα	Multiple spliced form	TPY	42,45

survival. Ras proteins are generally closely regulated by GEFs that promote GDP dissociation and GTP binding and GTPase-activating proteins (Zhang & Liu, 2002). Also, the RAS super family of GTP hydrolysis-coupled signal transduction relay proteins is divided into RAS, RHO, RAB, and ARF families, as well as the closely related Gα family (Zhang & Liu, 2002). They are mainly concerned with the control of cell proliferation, working as cellular switches, linking signals from receptors of the membrane to the expression of a particular gene. The classical mutations in the RAS proteins that are found in human tumors increase the amount of protein produced in the GTP-bound state, making them constitutively active (Zhang & Liu, 2002). Figure 2 showcase the signaling pathways and inhibitors of EGFR. Activation of EGFR leads to homodimerization/heterodimerization, and phosphorylation of specific tyrosine residues.

The majority of cancers occur due to the mutations that affect the signaling pathway involved in MAP kinase in the RAS and RAF genes (Yarden & Ullrich, 1988; Boulton *et al.*, 1991; Kaplan *et al.*, 1991; Robinson & Cobb, 1997; Zhang & Liu, 2002; Scaltriti & Baselga, 2006; Li *et al.*, 2017). Generally, even point mutation can cause drastic changes in the pattern of the on-and-off mechanism involved in that pathway and can result in an adverse impact on the body, which can be fatal (Quilliam *et al.*, 2002; Colicelli, 2004).

The RAS protein/RAS family of genes has three sub-categories which are:

K RAS:

- It is a GTPase protein and one of the early players in the signal transduction pathway. It is usually adhered to the cell membrane because of the isoprene group at its C terminus. It plays an important role in the conversion of GTP to GDP (Kaplan *et al.*, 1991; Quilliam *et al.*, 2002; Zhang & Liu, 2002; Colicelli, 2004).
- A high frequency of mutations of KRAS is reported in certain diseased conditions found in the pancreatic, large intestine, biliary tract, small intestine, lung, endometrial and ovarian cancers (Kaplan *et al.*, 1991; Quilliam *et al.*, 2002; Zhang & Liu, 2002; Colicelli, 2004). Table 2 demonstrates the frequency of mutations.

N RAS:

- This also serves as a GTPase and aids in transmitting signals by attaching a molecule of GTP. It functions in the maintenance of cell division, cell differentiation and apoptosis.
- The mutations of N RAS are associated with melanoma, nervous system, hemopoietic, lymphatic, and thyroid cancers.

H RAS:

- Its structure and adherence nature are similar to that of the KRAS gene protein. It also acts like a molecular switch playing on and off and hence activating the proteins required for the propagation of receptor signals, such as C RAF and PC kinases. It binds to GTP and possesses an enzyme cleavage activity at the terminal phosphate of nucleotide (Quilliam *et al.*, 2002; Zhang & Liu, 2002; Colicelli, 2004).
- The mutation in this gene can cause salivary gland, urinary tract, cervix, upper aero digestive tract, prostate, and skin cancer (Boguski & McCormick, 1993; Quilliam *et al.*, 2002; Zhang & Liu, 2002; Colicelli, 2004).

The mutations in the HRAS region result in various cancers among which the predominantly occurrences are discovered in the bladder (9%), upper digestive tract (6%), skin, and cervical cancer (16% and 5% respectively). RAS mutations commonly occur at G12 and G13 residues, which are responsible for the prevention of the binding between RAS and GAP thereby

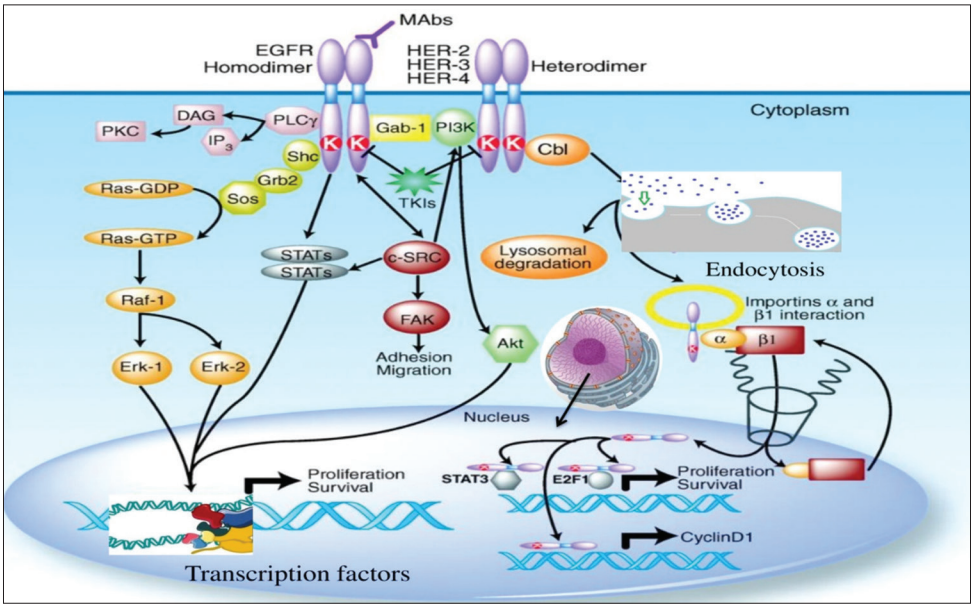

Figure 2: Signaling pathways and inhibitors of EGFR. Activation of EGFR leads to homodimerization/heterodimerization, and phosphorylation of specific tyrosine residues

Table 2: Frequency of Mutations

S. No.	Disease	KRAS (%)	NRAS (%)	HRAS (%)	BRAF (%)
1	Breast	4	2	1	1.1
2	Brain	1	1	0	8
3	Cervix	7	2	5	9.9
4	Endometrium	14	0	1	0
5	Lung	17	0.6	0.5	2.2

preventing the hydrolysis of GTP (Boguski & McCormick, 1993; Davies *et al*., 2002). Thus, these mutations occur in the active state leading to the activation of the subsequent downstream effectors and are referred to as hyperactivation.

Thus, RAS-RAF-MAPK-ERK pathways activate other effect or pathways like the phosphatidylinositol 3 kinase (PI3K) (Tsai *et al*., 2006). Research in several aspects has also led to the understanding that the activation of the MAPK signaling pathway could significantly promote the generation of epithelial-mesenchymal transitions (EMT) in cervical cancer cells (Lee *et al*., 1996). Hence, the activation of these pathways is imperative since it provides a cytoprotective tumor environment, consequently granting the tumor cells resistance to the conventional theory. This hypothesis can lead to new facets that are yet to be discovered and thus, subsequent discoveries can aid in the development of potential therapeutics.

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