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EDITORIAL

Hypocretin (orexin) pathology in Alzheimer's disease

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Abstract

Alzheimer's disease (AD) is a growing health problem. It has enormous public health impact. Sleep problems show an early component of this disease. Hypocretin has a major function in sleep-wake cycle. The total number of hypocretin neurons in the normal humans ranges from 51000-83000, located exclusively in the hypothalamus. Deficiency in hypocretins neurotransmission results in narcolepsy, Parkinson's disease, and other neurological and psychological disorders. Cerebrospinal fluid (CSF) hypocretin levels were directly related with t-tau protein amount in AD. Increased hypocretin CSF in AD suggest that hypocretin is involved in the mechanism of AD pathology.

Key words: Hypocretin; Orexin; Alzheimer's disease; Neurological disorders

Core tip: Hypocretin plays an important role in the control of sleep-wake cycle. Increased hypocretin levels in Alzheimer's disease patients suggest hypocretin system is involved during development of the disease symptoms.

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INTRODUCTION

The hypocretins were discovered in 1998 by two groups[1,2]. One group named hypocretins because of hypothalamic origin and similarity with the secretin[1]. The other group named Orexins because these neurotransmitters stimulated food intake[2]. Their projection target suggests hypocretins have a neuromodulatory role in neuroendocrine and homeostatic functions[3,4]. The distribution of hypocretins neurons in human hypothalamus is shown in Figure 1. Hypocretin fibers and receptors are found throughout the brain[3,5,6]. Hypocretins

Table 1 Important findings shows role of hypocretin in Alzheimer's disease

Ref.	Year	Major findings
Friedman *et al*[28]	2007	Increased wake fragmentation found in those with lower hypocretin-1
Kang *et al*[29]	2009	Amyloid-beta dynamics are regulated by hypocretin and the sleep-wake cycle
Fronczek *et al*[27]	2011	40% hypocretin cell loss in Alzheimer's disease
Slats *et al*[30]	2012	Association between hypocretin-1 and amyloid-β42 cerebrospinal fluid levels
Roh *et al*[8]	2014	Modulation of hypocretin and its effects on sleep to modulate Aβ pathology
Liguori *et al*[7]	2014	Increased hypocretin level correlates with sleep disruption and cognitive decline in Alzheimer's disease

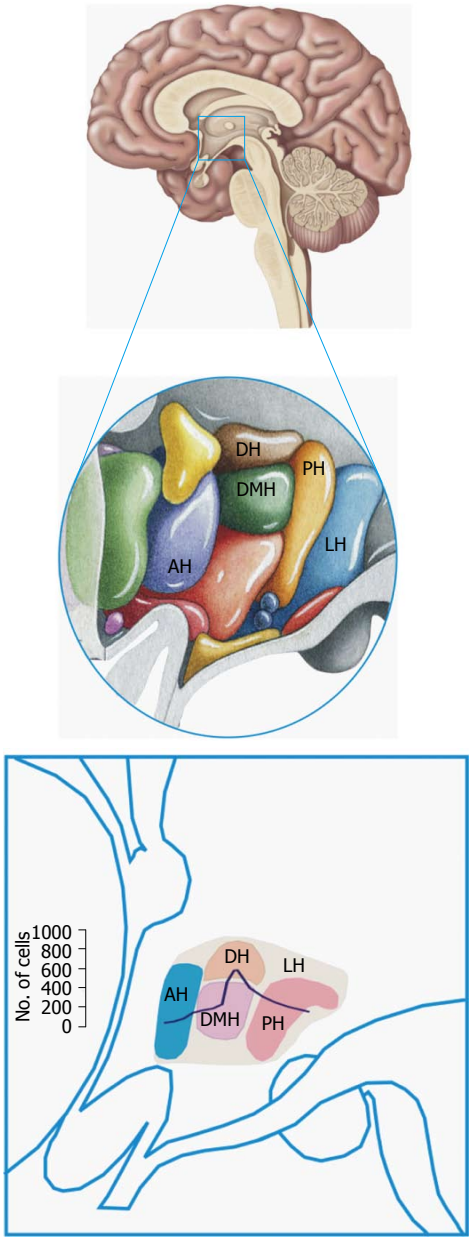

Figure 1 Distribution of hypocretin neurons in human hypothalamus. The normal distribution of hypocretins cells in the hypothalamus is limited to AH, DH, DMH, LH and PH. AH: Anterior hypothalamus; DH: Dorsal hypothalamus; DMH: Dorsomedial hypothalamus; LH: Lateral hypothalamus; PH: Posterior hypothalamus.

loss in narcoleptics opened importance of hypocretin system in health and disease[5]. New findings show the role of hypocretin in the pathogenesis of Alzheimer disease (AD)[7,8].

HYPOCRETIN AND NEUROLOGICAL DISORDERS

Narcoleptic patients have low or undetectable cerebrospinal fluid (CSF) hypocretin[9]. The pathological studies revealed 85%-95% loss of hypocretins cells in narcoleptics with cataplexy[10]. Maximum cell loss was occurred in the posterior and tuberomammillary nucleus[11,12]. Decreased CSF hypocretin were reported in, idiopathic hypersomnia, hypothalamic neoplasms and acute disseminated encephalomyelitis[13-17]. Higher CSF hypocretin were found in restless legs syndrome[18]. Lower hypocretin CSF were reported in patients with multiple sclerosis[16], Niemann Pick disease type C[19] and Whipple's disease[20]. Hypocretin cell loss was found in Parkinson disease patients[21,22]. Benarroch *et al*[23] reported 70% loss of hypocretins cells in multiple system atrophy patients. In Huntington's disease 30% loss of hypocretins cells occurred[24]. Bauman *et al*[25] found hypocretins cell loss in TBI patients with severe injury. There was reduced fluctuations of hypocretins CSF in depression patients[26].

DYSREGULATION OF HYPOCRETIN SYSTEM IN AD

Number of hypocretins cells in AD patients were reduced by 40%[27]. AD patients with lower hypocretins-1 showed increased wake fragmentation[28]. Kang *et al*[29] reported the role of hypocretins and sleep in amyloid beta dynamics. The link between mean amyloid beta 42 and hypocretins suggests a relationship between AD pathology and hypocretin disturbance. The important findings related to the role of hypocretins in AD are summarised in Table 1. With symptom progress AD patients had increased hypocretins levels. The hypocretin levels in AD were associated with tau protein and sleep impairment. Hypocretin output and function seem to be over expressed with disease[8]. A few literature reports showing that[30,31] there was no decrease in CSF hypocretin levels. These studies considered smaller samples including some cases, patients receiving psychiatric medications, which may influence hypocretin neuronal activity and output. Liguori *et al*[7], results are in contrast to Fronczek *et al*[27], study reporting decreased

hypocrein neurons and CSF levels in AD patients. This difference may be related to the fact that Ligouri *et al*[7], performed *in vivo* study, whereas Fronczek *et al*[27], were used pathological tissues from advanced AD patients. Roh *et al*[8], found that hypocretin knockout animals slept for longer time and lower amyloid-beta. These studies show the importance of hypocretin system in AD.

AD AND NARCOLEPY

The core sleep problems in AD and narcoleptic patients are partly resemble. Hypocretin may have an important function in the pathological mechanism of AD. In narcoleptic patients with cataplexy have 90% of hypocretins cell loss and undetectable level of CSF hypocretin. If hypocretins mediates AD symptom progress, narcolepsy patients should be protected against AD pathology. The neuropatholgical records of twelve narcolepsy with cataplexy showed that thirty three percentages of these narcoleptics had AD pathology which is comparable to the prevalence in general population[32]. This report shows that severe loss of hypocretin neurotransmitter does not protect from AD.

HYPOCRETIN AS A CSF BIOMARKER

Higher CSF t-tau protein levels mark the AD neurodegeneration. Increased t-tau levels represent a sign of rapid cognitive decline because they have been faster more pronounced neuronal degeneration, supporting the transition from early to more advanced disease stages[33]. CSF hypocretin levels were directly correlated with t-tau protein levels in AD[8,34] . This finding suggests that higher hypocretin levels may be related to rapid tau-mediated degeneration in AD. The pathogenesis of AD may therefore involve dysregulation of the hypocretins system, with over expression of hypocretins output and function.

CONCLUSION

With a rising prevalence of AD around the world, there is an urgent need to identify opportunities for prevention and treatment of the disease. Hypocretin may have a role in the pathological process leading to AD. The pathogenesis of AD may therefore involve dysregulation of the hypocretins system, with over expression of hypocretins output and function, manifested as sleep disturbance and associated with progressive neurodegeneration. Further studies on the importance of hypocretin during the process of AD could lead to new preventive and therapeutic findings.

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EDITORIAL

Human T-lymphotropic virus type 1-associated myelopathy/tropical spastic paraparesis: Clinical presentation and pathophysiology

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Abstract

Human T-cell lymphotropic virus type 1 (HTLV-1)-associated myelopathy/tropical spastic paraparesis (HAM/TSP) is a slowly progressive neurodegenerative disorder in which lesions of the central nervous system cause progressive weakness, stiffness, and a lower limb spastic paraparesis. In some cases, polymyositis, inclusion body myositis, or amyotrophic lateral sclerosis-like syndromes are associated with HTLV-1. TSP was first described in Jamaica in 1888 and known as Jamaican peripheral neuritis before TSP was related to HTLV-1 virus, the first retrovirus being identified, and the disease is since named HAM/TSP. There is no established treatment program for HAM/TSP. Prevention is difficult in low-income patients (*i.e.*, HTLV-1 infected breast feeding mothers in rural areas, sex workers). Thus, there is a need for new therapeutic avenues. Therapeutic approaches must be based on a better understanding, not only of clinical and clinicopathological data, but also of the pathophysiology of the affection. Consequently, a better understanding of existing or newly developed animal models of HAM/TSP is a prerequisite step in the development of new treatments.

Key words: Tropical spastic paraparesis; Human T-cell lymphotropic virus type-1; Polymyositis; Animal models; Retroviruses; Myelopathy; Human T-cell lymphotropic virus type 1-associated myelopathy; Pathogenesis

Core tip: Human T-cell lymphotropic virus type 1-associated myelopathy/tropical spastic paraparesis (HAM/TSP) is a slowly progressive neurodegenerative disorder in which lesions of the central nervous system cause progressive weakness, stiffness, and a lower limb spastic paraparesis. There is no established treatment program for HAM/TSP. Prevention is difficult in low-income patients. Thus, there is a need for new therapeutic avenues that must be based on a better understanding, not only of clinical and clinicopathological data, but also of the pathophysiology of the affection. Consequently, a better understanding of existing or newly developed animal models of HAM/TSP is a prerequisite step in the development of new treatments.

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INTRODUCTION

Human T-cell lymphotropic virus type 1 or human T-lymphotropic virus type 1 (HTLV-1), (also called the adult T-cell lymphoma virus type 1) is a retrovirus belonging to the family retroviridae and to the genus deltaretrovirus. HTLV-1 was the first identified human retrovirus and is the etiological agent of two distinct diseases: Adult T-cell Leukemia/Lymphoma (ATL) and HTLV-1-Associated myelopathy/tropical spastic paraparesis (HAM/TSP)[1]. It is estimated that HTLV-1 infects approximately 10-20 millions individuals worldwide[2]. Endemic areas include Japan, Brazil, the Caribbean, inter-tropical Africa, Eastern Europe, and the Middle East. In United States, Canada and Western Europe, it appears that HTLV-1 is mainly found in immigrants. It seems however that there is a cluster of HAM/TSP cases in Romania. One to 5 percent of the individuals infected with HTLV-1 eventually develop ATL, an aggressive malignancy of mature activated $CD4^+$ T cells, characterized by frequent visceral involvement, and opportunistic infections secondary to T cell immunosuppression. One to four per cents of patients infected by HTLV-1 will develop HAM/TSP[2]. TSP was first described in Jamaica in 1888 and was known as Jamaican peripheral neuritis before TSP was related to HTLV-1 in patients from Martinique presenting similar symptoms[1]; the affection was then called HAM. Large cohorts of patients with TSP have been reported in Jamaica[3]. HAM/TSP seems more common in lower socio-economic groups and more prevalent in rural regions. Currently, there is no established treatment program for this disorder. Prevention is difficult in low-income patients (*i.e.,* HTLV-1 infected breast feeding mothers in rural areas, sex workers). Even if the incidence is currently lower due to detection of HTLV-1-positive blood donors, there are still numerous cases of HAM/TSP.

CLINICAL PRESENTATION

Infection of HTLV-1 can be spread through contaminated blood products, passage from mother to child and sexual transmission. Very high rates of transmission of HTLV-1 are observed with blood products containing infected cells, as well as after transplantation of organs from HTLV-1 positive patients. However, blood screening prior to transfusion reduces the rate of transmission through infected blood products. Viral transmission can also occur through the use of injected drugs. Sexual transmission is mostly male to female, and explains the increasing numbers of HTLV-1 positive women, particularly in sex workers. Passage from mother to child can occur within the womb, by perinatal transmission, or *via* breastfeeding, particularly if prolonged after 6 mo of age, and if the proviral load is high in the milk. In Jamaica, HAM/TSP seem to be more frequent in rural areas and in low income patients[3].

HAM/TSP is a slowly progressive neurodegenerative disorder in which lesions in the central nervous system (CNS) predominate in the lower spinal cord and cause progressive weakness, difficulty walking, stiffness, and a lower limb spastic paraparesis. Foot dragging, difficulty running, impairment of ambulation, are present in 60%-80% of cases. Weakness usually begins on one side, then extends to the other side over months or years. Pyramidal signs, paraparesis and urinary symptoms are observed in almost 100% of cases. Legs are uniformly involved but arms are weak in up to 33% of patients. Hyperreflexia of lower limbs and Babinski sign are present in more than 90% of cases. Sensory signs (paresthesia and sensory cord levels) are less frequent (25%-78%). Pain and muscle atrophy are less common. Disability occurs during the first year of the disease. The disease is progressive without remissions. The WHO diagnostic criteria for HAM/TSP require presence of paraparesis (associated or not with sensory and/or autonomic abnormalities), positive serology for HTLV-1, and presence of the virus in the cerebrospinal fluid[4].

In addition to the typical HAM/TSP, there are some neurological manifestations related to HTLV-1, but without the typical form. Manifestations of autonomic dysfunctions (overactive bladder, heart rate and blood pressure dysregulation, erectile dysfunction, constipation), and sensory dysfunction (impaired proprioceptive and vibratory function) can be observed without HAM/TSP. Overactive bladder, characterized by incontinence, nocturnia, and urgency, is one of the most common symptoms in patients without paraparesis. In some cases, overactive bladder precede HAM/TSP by years, being the initial manifestation of the disease. Patients with neurological symptoms not fulfilling the typical HAM/TSP criteria can be classified as possible or probable HAM/TSP[4-6].

Other neurological symptoms linked to HTLV-1, but not related to myelopathy have also been reported. Cognitive dysfunction (impairment of visual and verbal memory, abnormalities of attention, and Mini-Mental State Exam), as well as cerebellar ataxia (mostly vermian cerebellar syndrome, frequently progressing to HAM/TSP, suggesting a spinocerebellar syndrome) have been associated to HTLV-1. Rare amyotrophic lateral sclerosis (ALS)-like syndromes have been described, but they differ from typical ALS by a long-term survival, and the presence of overactive bladder. Polyneuropathy, mostly sensory-motor polyneuropathy, can be associated to HTLV-1. Peripheral neuropathy (predominantly axonal) can be found in 30% of patients with HAM/TSP, but more rarely in patients without HAM/TSP (6%). Cases of cranial mononeuropathy,

usually facial nerve palsy, have been reported; a study in Trinidad and Tobago found that 21% of cases of facial nerve palsy were associated with HTLV-1[4-6]. In some cases, polymyositis, and inclusion body myositis, are related to HTLV-1, but are usually not associated with HAM/TSP. In Jamaica, 85% of patients with polymyositis are HTLV-1-positive[7].

Symptoms usually begin around age 30. Most patients have insidious course progressing over months to years. Median time from onset to use of a cane is 6 years, a walker at 13 years, and a wheelchair at 21 years[8,9]. About 10%-20% progress to severe gait impairment over 1-3 mo. More rapid progression tend to occur in patients older than 50 with a high viral load[8], or after blood transfusion[10], or organ transplantation[11]. However, onset and course are highly variable. Disease usually begins with asymmetric leg weakness and stiffness. Progressively, other leg becomes involved over months or years. Spasticity then becomes more pronounced and impairment of ambulation soon appears[6].

Routine CSF analysis may be normal or show various abnormalities. Glucose level is normal. Protein is elevated in up to 40% of patients. Cell counts are elevated in up to 57% of patients, consisting entirely of mononuclear cells[12]. Elevated intrathecal production of IgG (IgG index, oligoclonal IgG, or CSF IgG synthesis rate) occurs in 20%-85% of cases. Proviral load in CSF cells is higher than peripheral blood mononuclear cells[6]. Brain MRI abnormalities are frequently seen (from 25% to 80% of patients)[13]. T2 protocols are more sensitive to demonstrate lesions appearing as T2 hyperintense signal abnormalities. Lesions, frequently multifocal, are observed in subcortical, periventricular and deep cerebral areas. White matter lesions are also frequent in HTLV-1 carriers, and the lesions in this group are similar to the ones in HAM/TSP patients[13]. electroenceph-alogram (EEG), and pathologic evaluation have shown a widespread involvement of the CNS. Diffuse EEG abnormalities (poor organization or slowing of background activity to that bursts and/or spikes) have been reported in 64% of patients[14]. Evoked potentials, particularly somatosensory evoked potentials (SSEPs) are frequently abnormal[15].

Many disorders can be discussed in patients presenting with progressive or mildly relapsing myelopathy: multiple sclerosis, Lyme disease, vitamin B12 deficiency, human immunodeficiency virus (HIV) infection, spinal cord compression. Konzo is a form of spasticity prevalent in Africa associated with excessive consumption of cassava and chronic cyanide intoxication.

Besides HTLV-1, myelopathies are rarely related to viral infections. Rare cases of tropical spastic paraparesis are caused by HTLV-2. Double infection with HTLV-1/HIV-1 is not infrequent in areas with high prevalence of acquired immune deficiency syndrome[16]. Other viral infections are uncommon causes of acute myelopathies (*e.g.,* poliomyelitis, herpesviruses). Besides poliovirus, flaviviruses (including West Nile virus), enterovirus-71, and cocksackieviruses A and B can induced anterior horn cell necrosis[16].

However, residence in a high seroprevalence endemic area, history of transfusion exposure, or IV drug abuse, or working as a sex worker, is highly indicative of the disease. CSF with inflammation and intrathecal production of IgG, abnormalities of SSEPs, hyperintense T2 signals or spinal cord atrophy on MRI are suggestive as well. Other associated systemic manifestations (*i.e.,* persistent prostatitis, dermatitis, bronchoalveolitis) can lead to the diagnostic that will be confirmed by an immunoassay and Western-blot showing HTLV-1 specific antibodies[4-6,16]. The Western-blot analysis differentiates HTLV-1 from HTLV-2 infection. Study of the CSF is the mandatory step to confirm the diagnosis. Polymerase chain reaction on CSF cells will confirm the diagnosis of CNS infection and help to distinguish HTLV-1 from HTLV-2[4-6,16].

PATHOGENESIS

There are different theories regarding the disease hypothesis. The most widely accepted theory related to HAM/TSP is that of a virally induced, cytotoxic, demyelinating inflammatory process of a chronic and progressive nature affecting the spinal cord. The infection by HTLV-1 triggers an antigen-specific immune response towards the HTLV-1 antigen. Cytotoxic $CD8^+$ T-lymphocytes of the host's immune response release cytokines in an effort to fight the infection. These cytokines facilitate the migration of lymphocytes across the blood-brain barrier (BBB). Demyelination is brought as a result of bystander cell injury, probably by apoptosis of oligodendrocytes. Activated microglia are a prominent feature found in the spinal cord of patients with HAM/TSP[17]. However, the role of microglia is not totally clear. Cells of microglia/macrophage lineage might be one of viral reservoirs in the spinal cords in HAM rat disease[18]. However, this point is somehow still debated. Unlike most other viruses, cell-free HTLV-1 is poorly infectious and efficient infection requires cell-cell contact. In the brain of some HAM/TSP patients, astrocytes are infected with HTLV-1. However, HTLV-1 is primarily found in $CD4^+$ T cells. Although $CD4^+$ T cells are the major reservoir, other hematopoietic cells ($CD8^+$ T cells, B lymphocytes, monocytes, macrophages, dendritic cells) and microglial cells have been infected with HTLV-1[19-23]. There are conflicting reports concerning the potential of HTLV-1 to infect microglial cells[24,25]. In one patient co-infected by HIV-1 and HTLV-1, presenting with HAM/TSP and HAND, HTLV-1 was localized to astrocytes and HIV-1 to microglia/macrophages[26]. In an attempt to study Tax-induced production of cytokines in human microglial cells and astrocytes, transduction of these cells has been done by using lentiviral vectors stably expressing Tax (oncoprotein of HTLV-1) gene. Results show that Tax can up-regulate cellular proinflammatory cytokine expression profile in human microglial cells and human fetal astrocytes[27]. However, HTLV-1 specific $CD8^+$ lymphocytes that secrete the neurotoxic cytokines

interferon-gamma (IFN-gamma) and tumour necrosis factor (TNF) are present[28] and may be responsible for bystander damage[29,30]. Extracellular Tax released from infiltrating T cells could induce cytokine release by microglia and contribute to demyelination and inflammation in the absence of detectable virus[31]. Like in other neurodegenerative diseases, it is possible that, associated with neuroinflammation, oxidative stress plays a role in the pathogenesis of HAM/TSP.

If the pathogenesis of HAM/TSP is still unknown, more data are available concerning the events leading to ATL. The pathogenesis of ATL results from the malignant transformation of CD4-positive cells. Tax induces this transformation by binding to transcription factors to promote transcription of the proviral genome. However, Tax has many other effects from repressing genes involved in DNA repair and activation of apoptosis, to inhibition of proteins involved in tumor suppression[32]. The distribution of proviral integration site is different between asymptomatic carriers, HAM/TSP and ATL patients[33].

ANIMAL MODELS

A number of various animal models have been developed for HAM/TSP. However, none of these models fully recapitulates HTLV-1-associated disease. Injection of immortalized MT-2 cells infected with HTLV-1 has been used in numerous experiments. ICR mice have been immunized with HTLV-I carrier T lymphocytes (MT-2 cell line) and then inoculated intracerebrally with these cells. In this experiment, perivascular cell infiltration was observed diffusely throughout the brain for over 2 wk[34]. HTLV-I antigens were detected in both sides of the cerebral hemisphere and tissue damage consisting of demyelination, axonal degeneration, and astrogliosis was observed most heavily between days 10 and 14[34]. Intraperitoneal (*ip*) inoculation of immortalized MT-2 cells infected with HTLV-1 in newborn WKAH rats can induce a chronic progressive myeloneuropathy with spastic paraparesis linked to apoptosis of oligodendrocytes in anterior funiculi of upper thoracic spinal cord. However, these signs appear 15-22 mo after inoculation[35,36]. The same animal model is characterized by activation of TNF-alpha and pX (area of HTLV-1 genome where genes for Tax and Rex regulatory proteins are located) genes[37,38]. Mononuclear infiltration was seen in the animal model previously described. Activated microglial cells and macrophages were observed 15 mo after HTLV-1 injection in WKAH rats. IFN-gamma can protect against the development of HAM rat disease[39]. If all these results are important, they have been obtained mainly in WKAH rats, mostly injected *ip* few days after birth, and signs appear more than one year after inoculation of immortalized MT-2 cells infected with HTLV-1.

Intravenous (*iv*) injection of whole blood from HAM/TSP patients or cells infected with HTLV-1 gives conflicting results[40-42]. However, these findings support the human evolution of the disease with its expression during adult or older adult age, as observed in rats aged 12 to 15 mo, corresponding on a human scale to 40 to 60 years of age. Besides presenting tumors, Tax (oncoprotein of HTLV-1) transgenic mice can develop a disease characterized by degeneration of oxidative muscle fibers or symmetrical paraparesis of the hind limbs[43]. Inoculation of HTLV-1 in monkeys induced polymyositis[44].

New ways of inoculating HTLV-1 should be investigated. HTLV-1-infected MT-2 cells have been used so far; however, most of the work has been done in newborn WKAH rats injected *ip* with these cells and HAM/TSP appears after at least one year in this model. The BBB might prevent HTLV-1-infected MT-2 to reach the CNS. It has been shown that faster and more reproducible results were obtained in adult mice with a direct intra-cerebral injection of these cells[18], suggesting that BBB might be a critical factor. In fact, it has been shown that human endothelial cells can be infected *in vitro* by HTLV-1[45], and that co-cultures of HTLV-1 and with human brain endothelial cell line leads to loss of tight junction proteins[2]. However, these results have been obtained *in vitro*. One way to circumvent the BBB would be to cause a breach of it for example by using an *ip* administration of mannitol before injecting HTLV-1-infected MT-2[46]. Inoculating cells in the cisterna magna, an area close to the spinal cord, following *ip* injection of mannitol would be less traumatic and more simple than directly into the brain or lateral ventricle[47]. Alternatively, *iv* injection of whole blood of patients with prior administration of mannitol, could be realized[48]. Moreover, it has been shown *in vitro* that BBB is abnormal in HTLV-1 related injury, and the previously described models could mimic these features[2].

In conclusion, more studies are necessary to define the pathophysiology of HAM/TSP[49]. Better animal models can pave the way for novel therapeutic approaches.

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FRONTIER

Mechanical transduction by ion channels: A cautionary tale

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Abstract

Mechanical transduction by ion channels occurs in all cells. The physiological functions of these channels have just begun to be elaborated, but if we focus on the upper animal kingdom, these channels serve the common sensory services such as hearing and touch, provide the central nervous system with information on the force and position of muscles and joints, and they provide the autonomic system with information about the filling of hollow organs such as blood vessels. However, all cells of the body have mechanosensitive channels (MSCs), including red cells. Most of these channels are cation selective and are activated by bilayer tension. There are also K^+ selective MSCs found commonly in neurons where they may be responsible for both general anesthesia and knockout punches in the boxing ring by hyperpolarizing neurons to reduce excitability. The cationic MSCs are typically inactive under normal mechanical stress, but open under pathologic stress. The channels are normally inactive because they are shielded from stress by the cytoskeleton. The cationic MSCs are specifically blocked by the externally applied peptide GsMtx4 (aka, AT-300). This is the first drug of its class and provides a new approach to many pathologies since it is nontoxic, non-immunogenic, stable in a biological environment and has a long pharmacokinetic lifetime. Pathologies involving excessive stress are common. They produce cardiac arrhythmias, contraction in stretched dystrophic muscle, xerocytotic and sickled red cells, *etc*. The channels seem to function primarily as "fire alarms", providing feedback to the cytoskeleton that a region of the bilayer is under excessive tension and needs reinforcing. The eukaryotic forms of MSCs have only been cloned in recent years and few people have experience working with them. "Newbies" need to become aware of the technology, potential artifacts, and the fundamentals of mechanics. The most difficult problem in studying MSCs is that the actual stimulus, the force applied to the channel, is not known. We don't have direct access to the channels themselves but only to larger regions of the membrane as seen in patches. Cortical forces are shared by the bilayer, the cytoskeleton and the extracellular matrix. How much of an applied stimulus reaches the channel is unknown. Furthermore, many of these channels exist in spatial domains where the forces within a domain are different from forces outside the domain, although we often hope they are proportional. This review is intended to be a guide for new investigators who want

to study mechanosensitive ion channels.

Key words: Channel; Mechanical; Patch; Force; Tension; Bilayer; Domain; Osmotic; Transduction; Biomechanics

Core tip: Mechanosensitive ion channels are found in all cells and their physiological function in most cells has yet to be defined inviting new researchers to the field. This review provides some guidelines to help newcomers understand key issues and potential artifacts.

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A WIMPY BACKGROUND

We are all familiar with many forms of mechanical transduction[1] including hearing[2], touch[3] and mechanical pain[4-7] that feed the central nervous system. In addition, there are the unconscious motor pathways bearing information about muscle stress and joint position[8,9]. Afferents in the autonomic nervous system service blood pressure regulation[8,10-12] and the distension of hollow organs[13,14]. While these reminders may be familiar to the readers of this journal, what may be less familiar is that all cells in our body, including red cells[15,16], are mechanically sensitive[17]. This cell sensitivity probably reflects our evolutionary origins[18]. Single cell prokaryotes like Paramecia have differentiated touch senses; if they bump into walls they back up, and if you bite their tails they run away[19-24]. We have the same heritage, but what are these sensors?

Mechanical sensitivity requires that forces do work on the sensor. This means that the sensors must be deformable, but that only narrows the field to all molecules since they can bend and stretch; it's a quantitative issue. Computational biology of molecular dynamics shows molecules moving under thermal and externally applied forces. How does one design a mechanical sensor? It's a matter of numbers. Sensors have a big output energy compared to the input energy. I will define mechanical sensors as those molecules or organelles in which the output energy, whatever that may be, is significantly larger than the input energy. Ion channels can do this because they are enzymes that dissipate lots of stored energy by catalyzing ion transport and their output energy is a function of the turnover number which can be 10^7.

All sensors have a background noise due to the random shaking of molecules (thermal fluctuations). In this review I will focus on ion channels as sensors (since that is my background), but all structural molecules can be viewed as mechanical sensors since they are compliant to applied force. The channels serve to couple mechanical stress to electrophysiology and biochemistry, typically by changes in calcium levels. This coupling of biochemistry to mechanics is familiar in muscle contraction, mechanically induced changes in gene expression, stem cell differentiation and changes in cell shape. We conjecture that all pathologies involve mechanics since they all involve a change in cell shape and that requires changes in force.

I will define mechanosensitive channels (MSCs), mechanically sensitive ion channels, as those channels whose dynamic range is fully accessible with physiologically relevant forces[25-37]. Many other ion channels, such as the voltage gated ion channels[38,39], are modulated by mechanical stress, but cannot span their dynamic range with mechanics alone. Many enzymes are also mechanically sensitive[40]. There are two basic types of MSCs: those gated by stress in attached structural proteins, and those gated by tension in the bilayer. MSCs in the differentiated sensory organs seem to be gated by forces in structural proteins[30,41-45] where the channel is in series with the extracellular matrix and the cytoskeleton (Figure 1). The other class of MSCs is gated by tension in the lipid bilayer (Figure 2)[24,42,46-50].

There is currently one drug known specific for MSCs of any kind, a peptide called GsMtx4 that is active on cation selective, bilayer-activated, MSCs[15,25,51-58]. Recently there has been a report of an organic molecule that tends to activate some of these channels[16].

This review is not intended to serve as a guide to the literature of MSCs, but addresses core issues that are required to understand how they work. For access to the general literature, there may be more review articles than research papers[18,24,42,46,47,59-63]! My primary goal is to familiarize newcomers to the field about what we have learned and to warn readers about some misleading dogmas to sensitize readers to critical interpretations of research papers. Many of the references in this paper come from the work in my lab, but that is not to say that they are the most important available, merely that I remember them better.

DEFINITION OF THE STIMULUS IN SIMPLE SYSTEMS

Lipid patch

One of the critical limitations of work on MSCs is that the stimulus tends to be imprecise. We can pull, indent, swell or shrink a cell, but what does the channel feel? We don't know, although but we can guess from the channel behavior. Even in plain lipid bilayer in a patch[46,47,61,64-66], we don't know the stresses with precision. Let's look at what might be considered the simplest of experimental systems: a patch of a liposome (Figure 3). Patches stick to the glass pipette. This adhesion produces a tension in the membrane that is a significant fraction of the lytic tension. This means that no one has ever recorded

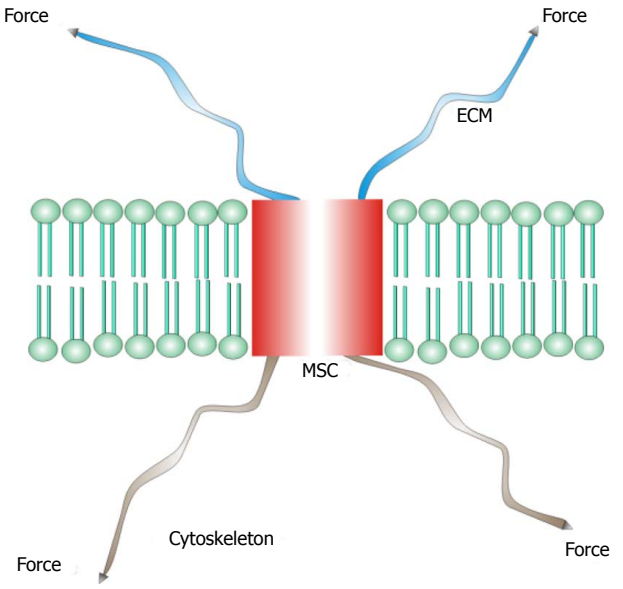

Figure 1 A mechanosensitive channel from a differentiated sensory organ where stress is passed through the channel from the extracellular matrix to the cytoskeleton. MSC: Mechanosensitive channel; ECM: Extracellular matrix.

patch currents of any kind except under extreme, nonphysiological, tension (there is one exception to this statement; we were able to measure MSC kinetics under low stress for short periods of time[67]). Patch tension is far in excess of anything seen in resting cells[31,33,68-70]. We know that tension can modulate many ion channels[38,39,67,71], probably transporters[72] and other membrane bound enzymes. When you read a paper about patch clamp recording, remember that all data was obtained under extreme membrane tension[59] and the patch data may not apply to the same channels *in situ*.

Returning to our ideal lipid patch, without pressure applied to the pipette the patch is pulled flat by adhesion to the glass. When we apply pressure, the patch bends, and the average tension in the membrane can be calculated from Laplace's law that states that the tension $T=Pr/2$ where P is the pressure across the membrane and r is the radius of curvature of the patch (not the radius of the pipette)[50,73-77]. From an image of a patch we should be able to calculate the membrane tension, and thus the stimulus that drives an embedded channel. Not so fast… notice that only the outer monolayer of the bilayer touches the glass. The inner monolayer is floating on the outer one[78-81], and as a fluid it cannot have any tension gradients at equilibrium[82,83].

Thus, even our simplest system has a gradient of tension normal to the membrane. You will notice that the papers on molecular dynamics simulations of MSCs apply a uniform tension across the bilayer in incorrectly compare those results to patch clamp data. While the mean tension in a membrane is related to the pressure across it by Laplace's law, models of membrane patches ignore the high membrane curvature where the patch contacts the glass (Figure 3) at the upper end of a seal[44,68,84,85].

Planar bilayer

We might imagine that an even simpler experiment that gets rid of the glass, a planar bilayer where the membrane is floating in space[66,86-90]. However, these bilayer lipids are attached to a support structure such as a hole in a Teflon partition. Lipids wet the support, and this adherence creates significant tension (approximately 5 mN/m) in the bilayer (the bulk lipid lining the hole in the support is known as the Plateau-Gibbs border[91,92], Figure 4). No one has made a measurement of channel activity in an unstressed planar bilayer[66,93], and for MSCs, this is a serious bias. The bilayer experiments of Coste *et al*[66] on the PIEZO channels suffer from this bias. If one is working with channels that inactivate, the resting tension in membranes, either in planar bilayers or patches, can put channels in an inactivated state making them invisible to a patch recording, and making a reconstitution experiment appear to have failed[18,43,48,94,95].

New method

There is one method, not currently in use, that might solve some of these problems. If the channels are reconstituted into large lipid vesicles (approximately 10 μm in diameter), and the vesicles are patched in "whole-cell-mode" then most of the membrane is not in contact with glass. By controlling the pressure in the vesicle which is large enough to measure accurately the radius of curvature, Laplace's law will provide reliable estimates of the mean tension and there won't be significant tension gradients.

STIMULATING CELLS

While lipid membranes are simple model systems, they are not cells. Cells are not homogenous solutions of macro molecules, but heterogeneous, anisotropic, viscoelastic/plastic structures made of multiple proteins and lipids with an extracellular matrix[43] that can carry stress between the exterior and the interior of the cells. But let's ignore these details for a moment and think of how we to experiment on cells.

Direct mechanical stimulation

The simplest stimulus is to poke a cell, usually with a fire polished pipette[93]. What does that do to MSCs? Imagine pressing a chopstick against a clump of Jello representing the cell interior. There is an indentation (more formally called a displacement) at the site of stimulation, but the amount of displacement decreases with distance from the site of stimulation. The stimulation is not uniform even at this macro level. This variation of stress/strain with distance occurs whether one stresses the cell with a pipette, a magnetic bead[96], a bead in a laser trap, an atomic force cantilever[97,98] or local perfusion[99]. It's

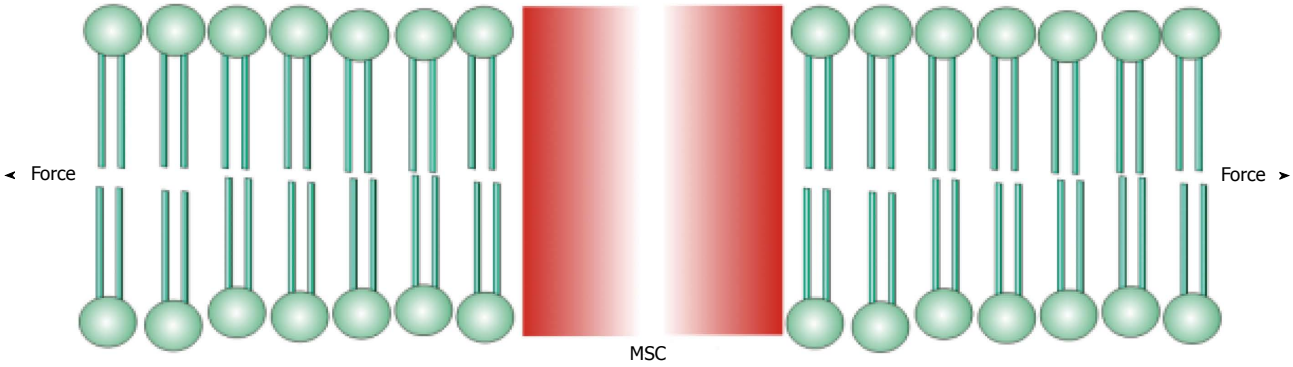

Figure 2 A mechanosensitive channel activated by tension (force) in the bilayer. MSC: Mechanosensitive channel.

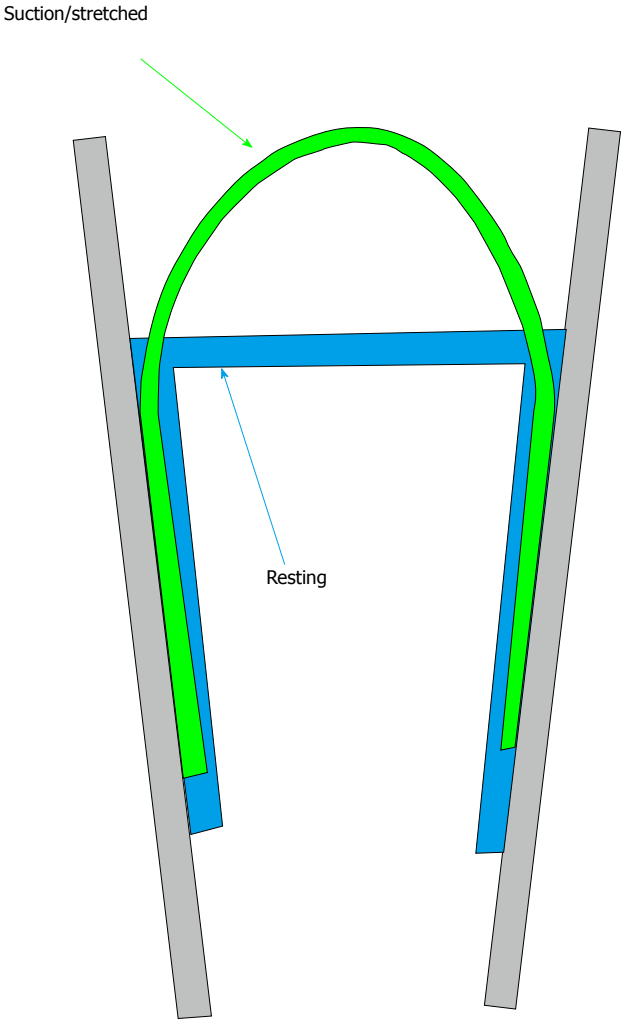

Figure 3 Cartoon of a patch pipette holding a lipid membrane at rest (pulled tight by adhesion to the glass) and under suction. Notice that suction peels the membrane off the glass a bit and functional channels.

just a property of a deformable material[100]. When you record mechanically-induced currents from a cell, the response is represents a mean value from a distributed stress, and it decreases nonlinearly with distance but covers larger and larger areas[100]. The best you can hope for is that the response is proportional to the stimulus, and hopefully monotonic. Further complicating

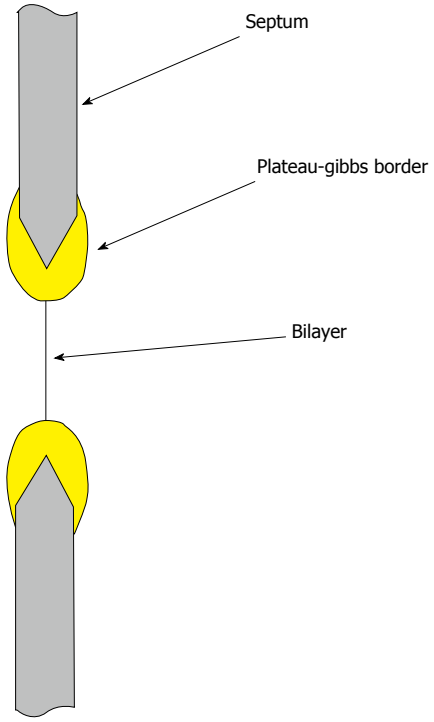

Figure 4 The planar bilayer is under tension, approximately 6 mN/m due to adhesion of the bulk lipid to the septum support.

those assumptions is the fact that the cell is viscoelastic and plastic - local stresses change with time even with a constant stimulus. Modelling of channels *in situ* usually assume the channels are uniformly distributed, but this is a bad assumption since, in general, they aren't (Figure 5). Remember to remain humble when interpreting your data since you really don't know the details of the stimulus.

In case I haven't yet scared you away from the field, let's look more closely at real cells. We will stick with whole-cell recordings where the local effect of the pipette glass is not significant. The key problem is determining what the channel feels. The ability to reconstitute bacterial MSCs in lipids shows the channels respond to tension in the bilayer and don't interact with a cytoskeleton, and that seems to apply to PIEZO MSCs as well[43]. What is the actual tension in the bilayer when

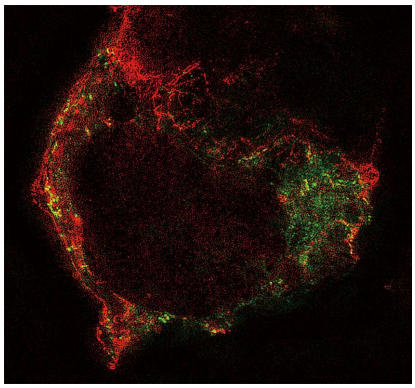

Figure 5 Structured illumination image of an human embryonic kidney cell cotransfected with human PIEZO1 channels labelled green and TREK-1 channels labelled red using green fluorescent protein mutants. Notice that the channels are in different structural domains and thus feel different forces. Notice also that TREK-1 tends to follow underlying cytoskeletal fibers. (Courtesy Gottlieb P).

MSCs are activated in cells? No one has measured it. The bilayer is supported by the cytoskeleton that shields the bilayer from excess stress (known as "mechano-protection"[36,101-103]). One experiment dealt with the distribution of stresses between the cytoskeleton and the bilayer in patches of an human embryonic kidney (HEK) cell, it there it was about 50:50. The cytoskeleton can thus alter the stress in the bilayer. Defects in the cytoskeleton can lead to diseases like muscular dystrophy[104-112].

Laser trap measurements of bilayer tension in resting cells suggests that it is negligible[69,70,113-115]. That fits our common observation that MSCs are not active in resting cells (Figure 6)[53]. Why do cells make MSCs if they can't be activated at normal stresses? Cortical stress is shared between the cytoskeleton and the bilayer, so bilayer stress reacts to cytoskeletal stress and *vice versa*, and these stresses are time dependent. The effective viscosity that makes responses time dependent arises from viscosity of the lipids and the dynamics of bonds in the cytoplasm[76,94,111,114,116-119]. The existence of connections between the bilayer and the cytoskeleton mean that any drugs that affect the cytoskeleton are likely to affect MSC activity, although drugs rarely are tested for these effects.

Adding to the complexity of defining the stimulus, lipids will flow under stress, and membrane lipids are not even homogeneous[46,76,116,120]. Spatial domains do exist[120-122] and physics tells us that the stress outside a domain is different from the stress inside a domain[73-75,123]. The energy gradient of stress at the edge of a domain is known as line tension. That affects the force inside the domain relative to that outside[75]. While we don't know in detail the stimulus at an MSC, we are safe in assuming the stress is greater than zero and less than the lytic limit of the bilayer.

If the transmembrane domains of a channel are thicker or thinner than the surrounding bilayer, the bilayer will bend at the boundary and those stresses are likely to modify MSC activity[124]. This is termed a hydrophobic mismatch, but the local curvature does not extend more than a few lipids from the channel[90,125-127]. However, amphipaths can dissolve in the membrane[128-131] and interact locally with the channel modifying the local stress and affecting channel gating. For example, the general anesthetics at clinically relevant concentrations cause opening of two-pore domain K^+ selective TREK-1 channels[132]. Opening these channels hyperpolarizes neurons possibly accounting for general anesthesia. The presence of these channels may explain why people can be knocked out by a blow to the head.

Osmotic stimulation

Suppose instead of these local mechanical stimuli we try for a more uniform stimulus like hypotonic stress? Cells swell with hypotonicity and we have been taught that swelling will stretch the membrane. If cells were spherical objects with a fluid cortex like red cells, that could work, but nucleated cells are filled with cross linked gels and the gels are what store most of the osmotic stress[133]. Consider the basic mechanics. Cells are not spherical so there are forces normal to the membrane. Secondly, with a given pressure across the membrane, the tension will depend upon the local radius of curvature (according to Laplace's law), and cells do not have uniform curvature. But a more serious problem is that nucleated cells have a cytoskeleton that acts like a sponge, a three dimensional object that fills the cell volume. The mechanics of three dimensional objects are different[134] from those of two dimensional objects like membranes[98].

We found that osmotic swelling doesn't make the membrane tense unless the cytoskeleton is disrupted[98], contrary to my intuition and years of textbook dogma. In fact, swelling tends to make cells softer[98]! How can that be? It turns out that everyone has done the experiment. When we pick up a dry kitchen sponge it is stiff. If we put it in the sink, it swells and soaks up water and it becomes softer. What is magic about a sponge? Nothing. It is just a set of cross-linked wettable polymers just like the cytoskeleton[26,135,136], and cells presumably can move water the same way without the need to move solutes. The cell membrane still remains the rate limiting step for water movement, but most of the energy from an osmotic gradient is in the cytoplasm and not in the membrane[133].

We visualized the distribution of osmotic stress in the cytoplasm using genetically coded optical stress sensors placed in structural proteins[26,27,29,30,119,137-139]. This three dimensional cross linked structure allows cells to withstand huge osmotic pressures[69]. (Ask yourself why sponges don't lyse.) Many cells, like bovine endothelial cells (BAECs), can withstand distilled water for hours and remain viable. The predicted osmotic (hydrostatic) pressure due to exposure of cells to distilled water is about 7 Atm, twice the pressure in a car tire. The cell' s stability under this huge gradient arises because the cell interior is glued together like a sponge. In the case of BAECs, this bonding allows the cells to face severe

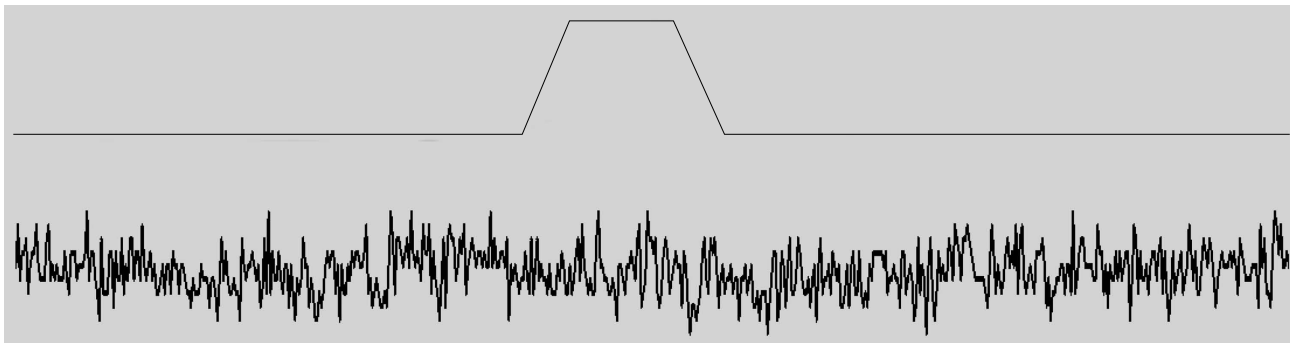

Figure 6 A whole-cell current recording (lower trace) from an human embryonic kidney cell in response to indentation of a few μm with a fire-polished micropipette in a piezoelectric manipulator (upper trace shows the cell indentation). There is no current associated with the indentation even though human PIEZO1 was cloned from the same cells! RNA expression does not mean the presence of functional channels, and even cells with no obvious endogenous currents may have channels, but they can't be activated because of mechanoprotection. They may become visible with large and/or repeated stimuli or treatment with agents like cytochalasin. (The trace is 1 s long and the RMS current noise is about 1 pA). RMS: Root-mean-square.

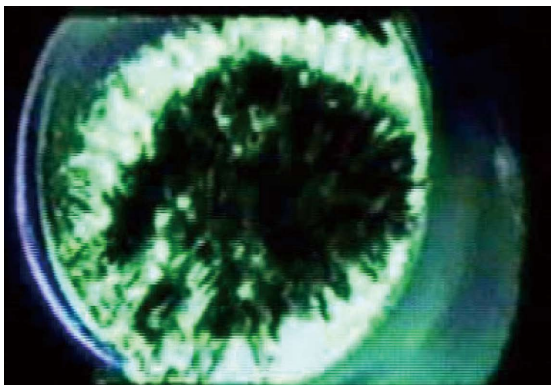

Figure 7 A frame from tomographic reconstruction of a patch of a Xenopus oocyte using high voltage electron microscope tomography[1]. The image shows cytoskeleton spanning the pipette and the bilayer is attached to the upper side but is not visible in this reconstruction due to its low density.

viscous drag in arteries where blood flow tries to rip the apical cortex from the basal cortex[27,140,141]. Osmotic stress does not stress the cell membrane very much, and despite many citations in the literature, osmotic stress should not be considered a "mechanical stimulus" of the cell membrane. Do not accept the results of papers that claim it is. Instead, treat those papers literally as dealing with the effects of osmotic stress.

There are a vast number of papers on cell volume regulation[55,98,142-147] invoking various ion channels such as the BK channels[148] and other K^+ channels[149], chloride channels[147,150-152] as well as neutral transporters[143,153,154] and water transporters[155-157] as well as the cytoskeleton[26-29,98,139,158] and host of calcium and other intracellular messengers[15,159-161]. Given the vast scale of modulators and potential effectors, it is unwise to think of cell volume as a specific stimulus.

Patch clamp stimulation

We all know about patch clamp recording and the revolution it created in our understanding of ion channels[162,163]. But what is a patch? The dogma says it is a bilayer containing channels[163] that spans the pipette. However, unless you are working with lipid vesicles, that is incorrect; patches are pieces of the cell cortex. Microscopy of patches (light microscopy[44,164] and electron microscopy[1,165-168]) show that patches are samples of the cell cortex, including the cytoskeleton (Figure 7) [1,165].

Whenever you make a patch, cell-attached or excised, the bilayer that contains your channels shares its stress with the cytoskeleton. How much does the bilayer feel in this composite structure? In the only published paper on the matter[169], we compared the amount of mechanical stress required to break a patch (pipette suction) with the voltage required to break the patch (typical of patch clamp "zap" voltages). The mechanical stress measured the lytic stress of the entire cortex. Voltage measured only the stress of the bilayer since that is where the voltage drop occurs. We measured the voltage required to lyse a patch as a function of the mechanical stress; the more mechanical stress, the less voltage. Since voltage only exerts force on the bilayer, we could separate the bilayer stress from the mean stress. It turns out that the bilayer lyses with a constant energy density, whether it comes from mechanics or voltage. For our particular cells, HEK-293, about half of the applied stress was in the bilayer and the rest in the cytoskeleton, but that result is from patches and we do not know how that applies to resting cells.

Regardless of the degree of stress sharing, no one has ever measured channel currents in a patch that emulates the tension characteristic of a resting cell[50,76]. The magnitude of the resting stress in a patch was emphasized to us when we tried to use used Triton-X100 to lyse patches. It doesn't work. The patches are stable. The reason is that detergents work by forming micelles. If you want to form a micelle in a patch under tension, you need to increase the membrane area since a plane plus a sphere has more area than the plane. The energy required to change the area of a membrane under tension T is $\triangle G = T\triangle A$ where $\triangle A$ is the change in area (Hooke's law in two dimensions). The energy available to the detergent is insufficient to form a micelle, but the

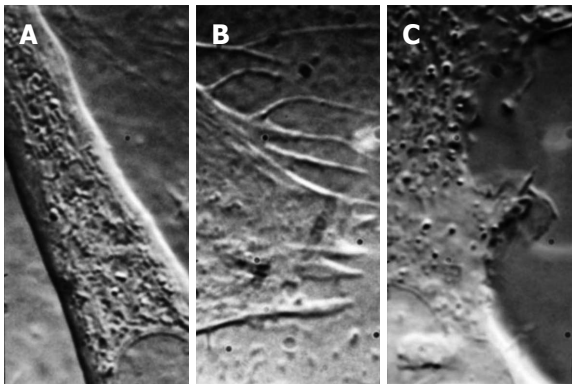

Figure 8 Differential interference contrast images of three different cell types (mouse myotube, left; rat astrocyte, center, human embryonic kidney cell, right) showing the variability of membrane structure and how patch clamp recordings are expected to be variable. The structures above change rapidly over time (this is a frame from a movie, Courtesy Suchyna T).

same detergent works well in the resting cell the patch because its membrane is not under tension.

Is the cytoskeleton of a patch the same as that in a cell? We don't know, but we do know that the chemical composition of a patch is different from that of the cell it was taken from[44]. We labelled different components of cell membranes and then patched them. We found that some elements made it to the pipette-spanning dome, and some didn't, notoriously the extracellular matrix[44]. That never even made it into the seal region. Some ion channels made it, and some didn't. You need to think of the pipette as a silica column. Biochemists know proteins stick to silica, so after dragging a membrane up a pipette, some things will stick to the glass and get filtered out, and some will make it to the dome.

The heterogeneity of the patch emulates the heterogeneity of the cell membrane and we know that the cell membrane is not homogenous (Figure 8). Even pure bilayers may not be homogeneous. Like ice and water, there are phase separations[170]. The amount of each phase (the fraction of total membrane area) is modulated by internal and external conditions. Cell membranes are much more complicated. If you look at a time lapse movie of cells, you will be impressed by the motion of the cell surface. Imagine your patch pipette coming down on one of these cells and then try to figure out which piece of membrane you patched. The answer, of course, is that you have no idea. Furthermore, given the data showing changes in patch composition with patch formation[44], and visible domains in a patch, you are in fact recording from a new mixture of cellular components. We suspect that patches might contain membrane from the endoplasmic reticulum and other organelles as well as the plasmalemma. Someone needs to check on that. We think that patch clamp recordings are as reproducible as they are because the formation of the patch helps to homogenize the components. In any case, be cautious about assuming that the properties of currents you get from a patch are the same as you would observe *in situ*.

WHAT CHANNEL ARE YOU RECORDING FROM?

We know that most if not all types of cells have endogenous cationic MSCs[15,17,51,55,128,171-174]. You may not see them frequently as they are normally closed because bilayer stress is shielded by the cytoskeleton ("mechanoprotection")[71,93]. You must know your background channel activity if you want to examine cells containing transfected channels. Treating the cells with cytochalasin or latrunculin to break up the cytoskeleton will reduce mechanoprotection and make background channels more visible[171]. Cell lines vary from lab to lab. According to the literature Coste *et al*[93] used N2A cells to clone PIEZO1, but Lee *et al*[71] used the same cell line and found no background PIEZO1 and 2 activity. Why? I expect that the cytoskeleton changes with passage number and with different batches of serum.

Because of the nearly universal presence of background channels, seeing a cationic MSC current after transfection does not mean you are seeing the channel coded by the DNA you used to transfect the cells. Furthermore, the expression of an MSC (or probably many other proteins) can cause massive structural changes in the cytoskeleton, even if the channel is non-conducting[175]. Thus, the process of transfection alone (not the effect of the transfection reagents, *per se*) can modify the forces that reach the channels.

We can now study cytoskeletal protein stress using genetically coded stress sensors[3,25,26,28-30,119,137-139,176]. The same issues apply to siRNA since suppression of one protein can affect others. For example, we showed that cytochalasin or colchicine affects the stress in actinin, spectrin and filamin and likely other structural proteins that are not judged to be the drug targets. When we modify any protein in a cell, we modify the stresses in the elements that are coupled to that protein.

Transfection can be a dangerous game. You can easily show modified RNA expression, we know that RNA expression is not cleanly related to the presence of functional channels. We cloned the human form of PIEZO1 and 2 from HEK cells[53], a human cell line of neural origin that usually exhibits little background MSC activity (Figure 6). The N2A cells that Coste *et al*[66] initially used to isolate PIEZO1, had no background MSC activity in other samples of the same cell line[71]. So how do we know what channels produced the currents we are looking at?

The best test would be to create a mutant channel with similar gating functions but with visibly different ionic selectivity than the endogenous channels. You cannot depend upon channel conductance alone[57] as a sound marker of expressed channels since it is easy to find situations in which the environment: cell-attached patch, inside-out or outside-out patch, or whole-cell, or planar bilayer have different conductances[66,177]. You would want a channel with big differences in selectivity, ideally a change from cation to anion selectivity!

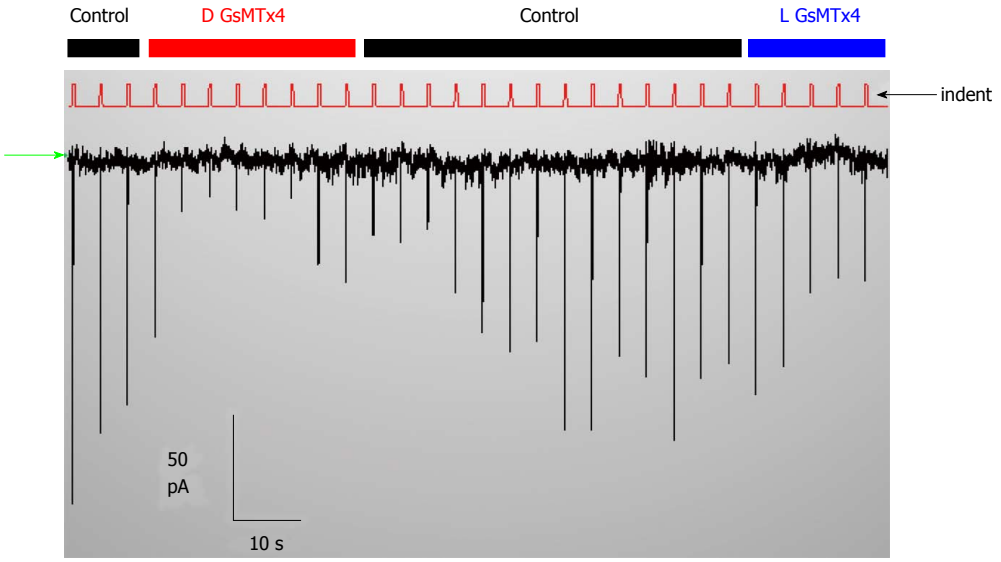

Figure 9 There are no PIEZO1 currents in this resting whole-cell recording unless the cell are indented (the red trace labelled indent shows the stimuli). The inhibitor GsMtx4 is effective in suppressing the stimulus-induced current but causes no change in the holding current. The stimuli are activated by computer control (www.qub.buffalo.edu). The baseline current (green arrow at left) is not affected by adding the D or the L enantiomers of GsMtx4, but the currents that are evoked by indentation are inhibited. Thus, tension in the resting cell is insufficient to activate PIEZO1.

You also cannot trust the channel kinetics as a marker since the kinetics of the channels depend upon their environment[177,178] and you have little control of that. Furthermore, you do not know if the channel you are trying to express might have a subunit that associates with an endogenous channel subunit or accessory protein, or induces the expression of previously unexpressed endogenous protein. It is well known that mechanical stress alters gene expression[179-182]. We know that expression of two different MSCs can create currents that do not belong to expression of either one alone[71]. There are no clean solutions in cells. We arbitrarily tend to look more closely at transfections that produce currents much larger than the background channels. We also don't know that we are looking at homomers of the transfected proteins, but we often make the simplifying assumption they are (this also aids in grant funding). Expression of green fluorescent protein labelled channels on the cell surface[43,48] or in patch membranes[44] does not mean that the fluorescent object is a functional channel, simply that the protein is present. We have tested the mechanical sensitivity of labelled transient receptor potential-canonical channels in patches and found they are present but are not mechanically sensitive[183].

WHY BOTHER WITH MSCS?

Cationic MSCs are normally protected from cell stress, so what do they do for a living? They do not seem to participate in behavior of normal hearts[184], but they do in stretched hearts where they seem to play a role in generating arrhythmias like atrial fibrillation[184]. They also play a role in muscular dystrophy where the channels produce a Ca^{2+} leak when the muscle is stretched[110,185]. We have come to believe that the typical cationic MSCs, like PIEZO1, serve primarily as sensors for potential bilayer failure. They would inform the cytoskeleton that the local bilayer is under excessive stress and likely to break, and the ion fluxes through the channel are signaling for mechanical reinforcement. The channels are functioning a bit like fire alarms whose function you unaware of until disaster looms. If the channels are closed in the resting cell you will not see the effect of inhibitory drugs like GsMtx4 on the currents (Figure 9). But if the same drug is active on open MSCs, you will see an effect, but to open the channels may require pathologic stress. Since channels like PIEZO inactivate, the information about the excess stress is transitory.

PIEZO1 mutations can cause anemias[43], and we have wondered why are these channels that inactivate quickly (< 30 ms) are present in red cells[186]. When does a red cell need such a short lived channel? We guess that the only time red cell stress becomes "pathological" is upon entry and exit from a capillary or perhaps a bifurcation. It might modify ion and water concentrations to reduce stress on the membrane as it is highly deformed upon entering the capillary. The same channels may be involved in sickle cell anemia where hemoglobin crystals push out on the membrane activating PIEZO1[15].

The universal presence of MSCs fits the common demand of all cells to avoid lysis and that occurs in disease. GsMtx4[187] and other agents that might act specifically on MSCs promise to be a new class of therapeutic agent with ideal selectivity they would only affect sick cells. We have found that GsMtx4 can be administered to mice daily for a month with no effect on behavior, and it can be injected into the CNS with no effect on behavior, but it does work to inhibit volume stimulated arrhythmias[188] and the phenotype of muscular dystrophy[109].

There is evidence that PIEZO2 channels may serve

a sensory role in nociception[4]. Since PIEZO was only cloned a few years ago, we have a lot more work to do. A nagging problem is why is PIEZO so big[189] - it is the largest transmembrane protein (approximately 2500 amino acids) and even tends to form tetramers with a MW of about 10^6 with the N and C termini about 20-30 nm apart[190] making us suspicious that PIEZOs have other functions; a large size is not necessary for MSC function[43,67]. There are many kinds of MSCs[191-194], nearly a dozen in bacteria alone, so we have lots of interesting problems to keep us busy.

CONCLUSION

This review has two goals, nominally for investigators new to the field of mechanotransduction: (1) Be humble about your data because you generally don't know your stimulus, and be explicit about your assumptions so people can read your paper properly. Quantitative models of the data have the intrinsic appeal of making the assumptions explicit; and (2) Create new preparations that can answer some of the pressing host of new questions.

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REVIEW

Molecular pathogenesis of glioblastoma multiforme: Nuances, obstacles, and implications for treatment

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Abstract

Glioblastoma multiforme (GBM), the literal apogee on the hierarchy of malignant brain tumors, remains one of the greatest therapeutic challenges in oncology and medicine. Historically this may be contextualized in the fact that the medical and scientific communities have had a very elementary understanding of its intricate and complex pathophysiology. The last 10-15 years have yielded a number of studies that have elucidated much of the molecular and genetic complexities of GBM that underlie its pathogenesis. Excitingly, some of these discovered genetic mutations and molecular profiles in GBM have demonstrated value in prognostication and utility in predicting response to treatment. Despite this, however, treatment options for patients have remained somewhat limited. These treatment options are expected to expand with the availability of new data and with the transition of novel treatment modalities from animal to human studies. This paper will have a threefold objective: provide an overview of the traditional paradigm in understanding and treating GBM, describe recent discoveries in the molecular pathogenesis of GBM against this historical backdrop, and acquaint the reader with new treatment modalities that hold significant therapeutic potential for patients.

Key words: Molecular pathogenesis; Temozolomide; Glioblastoma multiforme; Treatment resistance; Hypoxia; Recurrent glioblastoma multiforme; Bevacizumab

Core tip: This paper provides the reader with an overview some of the primary molecular markers that are implicated in the pathogenesis glioblastoma multiforme (GBM). It provides a robust review of the evidence that supports the use of these molecular markers for both prognostication and prediction for response to treatment. It gives the reader context for understanding the hypoxia model and how it informs treatment resistance in GBM. It provides an overview of cancer stem cells and their role in GBM biology. And it acquaints the reader with a few of the new, promising treatment modalities that are emerging.

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INTRODUCTION

Glioblastoma multiforme (GBM) belongs to a class of brain tumors known as gliomas, so named because they arise from glial cells (astrocytes, oligodendrocytes, ependymal and schwann cells). Glial cells have traditionally been understood as the workhorse cells of the central nervous system (CNS), providing the needful nutrients, oxygen and stromal support for neural cells. Recent studies have shown glial cells to have a more central and independent role in the CNS than historically thought, acting alongside neural cells in neurotransmission[1].

Since 1979, gliomas have been classified by the World Health Organization into 4 classes based upon histopathology, each successive class exemplifying features more consistent with malignancy[2] (Table 1). Indeed, of the 4 classes, only grades Ⅲ and Ⅳ are considered malignant gliomas due to possessing telltale histological features (increased cellularity, abnormally increased mitotic activity, nuclear atypia). On this hierarchy, GBM is classified as grade Ⅳ due to the typifying unique characteristics of ubiquitous neovascularization and dramatic necrosis of neoplastic tissue (due to the extent of cell turnover).

Epidemiology

GBM is the most common malignant brain tumor, and histologically is second in incidence only to meningiomas when considering all intracranial neoplasms, both malignant and benign. Based upon data compiled by the Central Brain Tumor Registry of the United States Statistical Report, GBM makes up 15.6% of all brain tumors and 45.2% of primary malignant brain tumors[3]. The incidence of GBM increases with age; highest rates are observed in 75-84 years old and, conversely, comprise only about 3% of brain and CNS tumors in 0-19 year olds. For reasons unclear, GBM is slightly more common in males.

Primary vs secondary GBM

GBM may be classified as either primary or secondary. As connoted by the names, primary GBM comes from native, wild-type glial tissue whereas secondary GBM comes about through malignant changes in lower grade gliomas (Grades Ⅰ and Ⅱ). When a case of GBM is diagnosed, determining whether it is primary or secondary is germane to the clinician for it allows him/her to make initial informed impressions on the biological and clinical behavior of the tumor, provides utility in prognostication and, increasingly, is guiding clinicians in predicting responses to molecular/targeted therapies. Before proceeding to characterize key genotypic differences between primary and secondary GBM, it is of interest to briefly delineate defining epidemiological and clinical features of these respective categories. Upwards of 90% of GBM cases are primary. These tumors are afflictions of the elderly, the mean age at diagnosis being 62. And they carry with them a uniformly poor prognosis at the present time, with roughly two-thirds of patients dying less than 3 mo from the time of diagnosis[4].

Secondary GBMs, by contrast, are predominantly cancers of a younger population, the mean age at diagnosis being 45. This tumor is characterized by a more indolent time course than Primary GBM, progressing from lower grade gliomas over the course of years as opposed to months[4]. Indeed, a population-based study from 2005 reported a mean time of 5.3 years to be the amount of time it took for low-grade astrocytoma to develop into GBM. In the case of anaplastic astrocytoma the mean time reported was 1.4 years[5]. Secondary GBMs represent a small minority of cases, accounting for less than 10% of total GBMs.

Primary GBMs have trademark molecular abnormalities that distinguish them from secondary GBMs, and it is these unique genetic aberrations that give each class the distinct characteristics discussed above (Figure 1). These are: mutations in the gene encoding the epidermal growth factor receptor (EGFR) protein that result in its amplification, loss of heterozygosity (LOH) of Chromosome 10q, phosphatase and tensin homolog (PTEN) deletion on Chromosome 10, and p16 deletion. Conversely, in Secondary GBMs, mutations of the ubiquitous p53 oncogene and of the gene encoding the platelet-derived growth factor receptor (PDGFR) protein are culpable for malignant transformation of lower grade gliomas[6]. A few of these molecular anomalies will be treated in detail in the paragraphs to follow (Tables 2 and 3).

Many of the gene products inextricably involved in the development of GBM are growth factor signal transduction proteins that transduce an extracellular signal *via* ligand binding into a cellular response. The cellular response regulated by these proteins is proliferation and growth. A very carefully orchestrated combination of positive and negative regulatory ligands in the extracellular milieu ensures that in the normal homeostatic state, growth and proliferation of glial cells is kept in check. A common recurring theme in malignant transformation is mutations that cause amplification or overexpression of the signal transduction protein products.

One of the best characterized signal transduction proteins that brings about malignancy in more than 40% of cases of primary GBMs is EGFR[4]. Among tyrosine receptor kinases, EGFR belongs to the ErbB receptor family, bearing significant genetic homology to three others-HER2/c-neu (ErbB-2), Her3 (ErbB-3) and Her4 (ErbB-4). The wild type function of EGFR is contingent upon binding a specific extracellular ligand

Table 1 World Health Organization classification of gliomas

Localized astrocytoma
WHO grade Ⅰ
Pilocytic astrocytoma
Pleomorphic xanthoastrocytoma
Subependymal giant cell astrocytoma
Diffuse astrocytomas/oligodendrogliomas
WHO grade Ⅱ (Astrocytoma)
Fibrillary
Protoplasmic
Gemistocytic
WHO grade Ⅱ (Oligodendroglioma)
WHO grade Ⅲ (Anaplastic astrocytoma)
WHO grade Ⅲ (Anaplastic oligodendroglioma)
WHO grade Ⅳ (Glioblastoma multiforme)
Giant cell glioblastoma
Gliosarcoma

WHO: World Health Organization.

Table 2 Primary glioblastoma multiforme *vs* secondary glioblastoma multiforme

	Primary GBM	Secondary GBM
Mean age at diagnosis	Approximately 62 yr of age	Approximately 45 yr of age
Percentage of cases	> 90%	< 10%
Clinical course	Rapid	Smoldering
Origin	*De novo*	Grade Ⅱ / Ⅲ astrocytomas

GBM: Glioblastoma multiforme.

Table 3 Hallmark genetic mutations, primary glioblastoma multiforme *vs* secondary glioblastoma multiforme

Primary GBM	Secondary GBM
EGFR overexpression/amplification	PDGFR overexpression
Loss of heterozygosity of Ch. 10q	Loss of heterozygosity of Ch. 10q
PTEN deletion on Ch. 10	p53 mutations
p16 deletion	p16/Rb pathway aberrancies

GBM: Glioblastoma multiforme; EGFR: Epidermal growth factor receptor; PDGFR: Platelet-derived growth factor receptor; PTEN: Phosphatase and tensin homolog.

at its extracellular domain, ensuring that it remains coordinated with physiological needs. On binding the ligand, inactive monomers of EGFR dimerize to an active form and provoke autophosphorylateion of the intracellular, C-terminal domain at multiple tyrosine residues. Certain intracellular signaling proteins bind EGFR and concomitantly activate signal transduction cascades. The end result is increased expression of genes that are involved in a pro-growth phenotype.

When mutated in GBM as well as other malignancies, the *EGFR* gene is typically amplified, in which case the protein is autophosphorylated constitutively and is thereby overactive.

However, in GBM there exists a unique mutation that generates a mutant protein Epidermal growth factor receptor variant Ⅲ (EGFRvⅢ) which is over-expressed. The mutation in the gene - a deletion of exons 2-7 -causes a deletion in the extracellular domain of the EGFRvⅢ protein that makes it inaccessible to extracellular regulatory ligands. This in turn leaves the protein in a constitutively active state that begets a slew of malignancy-specific features: cellular proliferation, the ability to invade other tissues, angiogenesis, and abnegation of the normal process of apoptosis. An interesting feature from the treatment perspective is that the deletion that yields the EGFRvⅢ protein encodes a codon that is not found in wild-type DNA and is unique to GBM[7]. Thus, conceivably, this sequence can be pursued as a specific molecular target in next generation treatment. In fact, studies are underway seeking to target EGFRvⅢ as a target. The phase Ⅲ ACT Ⅳ trial underway is investigating the cancer vaccine rindopepimut for this very purpose[8].

TREATMENT

Surgery

Whereupon magnetic resonance imaging all but cinches the diagnosis, the gold standard for confirmation remains tissue biopsy. Though this may be accomplished by stereotactic brain biopsy alone, tissue is more commonly procured with maximally safe surgical resection. As it stands, maximally safe surgical resection is by no means curative as by the time of diagnosis, the tumor has invariably insinuated itself deep into vital, surgically inaccessible tissue. This said, rote surgical resection does still provide the patient with relief from symptoms wrought by mass effect of the tumor. There is also demonstrable improvement in survival by resection of tumor burden, albeit marginal[6].

Radiation

Adjuvant radiotherapy has been an established cornerstone in the treatment of GBM since 1979, when publication of the seminal study by Walker *et al*[9] showed that patients treated with radiation showed longer survival than those treated with best supportive care.

A major obstacle in the radiotherapy of GBM is the problem of radiation resistance, which is recurrence of the tumor within the high dose region[7]. The existence of this phenomenon implies that the amount of radiation administered (and as tolerated without excessive toxicity) is not enough to eradicate in entirety tumor existing in the radiation field. It is hypothesized that some of the hallmark genetic mutations characteristic of GBM contribute to the phenomenon of radiation resistance. Studies that have looked at increasing the dose of radiation to the limit tolerated-up to 90 Gy-have not demonstrated a discernible benefit of this strategy.

Having discussed the limitations of current radiotherapy, it begs discussing new modalities being investigated that intend to overcome these limitations

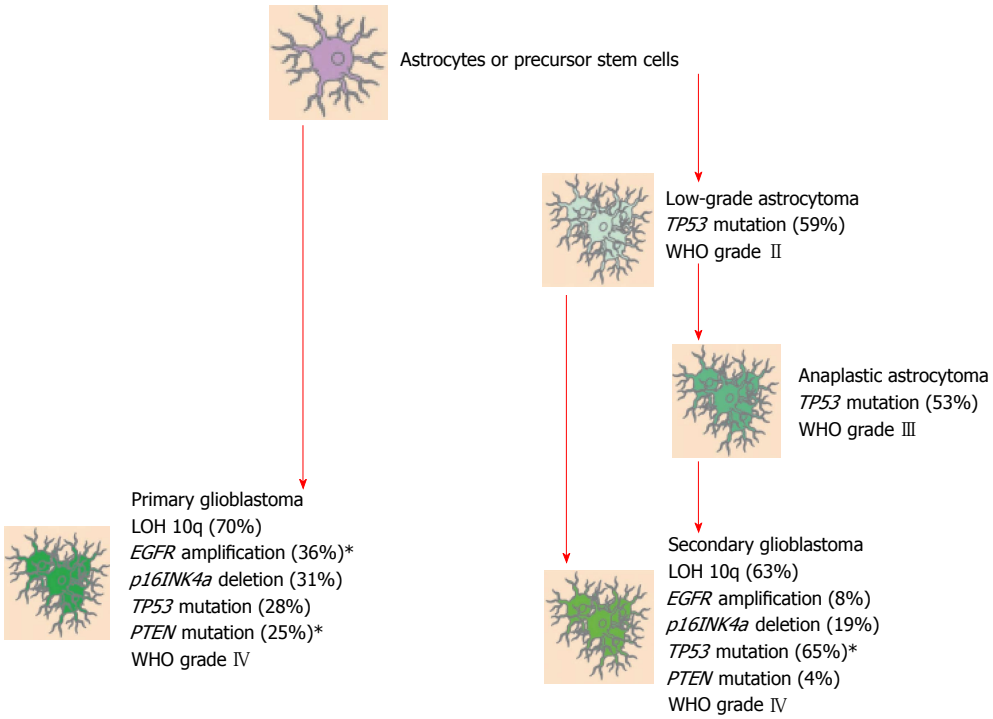

Figure 1 Genetic mutations pathways implicated in the development of malignant gliomas (76). WHO: World Health Organization; LOH: Loss of heterozygosity; EGFR: Epidermal growth factor receptor; *PTEN*: Phosphatase and tensin homolog.

which will be discussed later.

Chemotherapy

The chemotherapeutic agent temozolomide has been available since 1999. Pharmacokinetically, temozolomide is an oral agent with effective absorption and excellent bioavailability. Temozolomide is metabolized into 5-(3-methyltriazen-1-yl) imidazol-4-carboxamid. The therapeutic potential of temozolomide lies in the alkylation/methylation of the DNA of tumor cells, typically occurring at the N-7 or O-6 positions of guanine residues. Methylation causes irreversible DNA damage which in turn provokes tumor cell death[10,11].

The use of temozolomide as standard of care adjuvant chemotherapy for GBM patients is largely the result of a seminal trial by Stupp *et al*[12] in 2005. This randomized controlled trial compared the use of irradiation alone to the use of concurrent radiation and temozolomide chemotherapy followed by 6 cycles of adjuvant temozolomide. In the experimental group, concurrent temozolomide was administered at 75 mg/m^2 daily during irradiation (both arms received 30 fractions with total dose of 5500 to 6000 cGy) followed by 6 cycles of temozolomide 150 mg/m^2 (days 1 to 5 of cycle 1) then 200 mg/m^2 on days 1 to 5 of cycles 2-6, repeated on day 29.

It was found that temozolomide improved median overall survival (OS) (14.6 mo *vs* 12.1 mo), 2 year OS (27.2% *vs* 10.9%), 3 year OS (16.0% *vs* 4.4%), and 5 year OS (9.8% *vs* 1.9%). These statistically significant results corroborated the superiority of temozolomide and that continuously improved over time.

MOLECULAR MARKERS WITH CLINICAL RELEVANCE IN GBM

MGMT and chemotherapy resistance

It is of importance to reconcile the positive results of temozolomide use in GBM treatment with the fact that, as discussed at the beginning of this paper, the overall outcomes in patients with GBM still remain unequivocally poor. One important concept that helps to explain this in part is chemotherapy resistance and in the case of temozolomide, through damaged DNA repair.

These chemotherapy resistant cells express a protein, O6-alkylguanine DNA alkyltransferase (AGT), encoded in humans by the O-6-methylguanine-DNA methyltransferase (*MGMT*) gene. The AGT protein removes the alkylated moiety on the O6 position of guanine and renders the therapeutic modality of temozolomide obsolete. It has been found that there exists an epigenetic variant of tumor cells that are able to circumvent this mechanism of chemotherapy resistance. These tumor cells possess a protein that is responsible for methylation of the MGMT promoter; this methylation serves to silence the *MGMT* gene. As a result, such tumors are thought to be more sensitive to temozolomide. Based upon this, this molecular marker for MGMT methylation has been investigated as a means of predicting response to treatment with temozolomide. As early as 2005, the group of Stupp *et al*[13] recognized the implications of this gene in therapy and conducted a retrospective analysis on the tumors culled from subjects in their pivotal study establishing superiority

of adjuvant chemotherapy and radiation to radiation alone. For 203 patients whose tumors were found to possess the *MGMT* methylation gene, a substantive difference was found, with the progression free survival (PFS) being substantially greater in the experimental arm receiving temozolomide with radiation therapy (RT) than the control group receiving RT alone. On the basis of these findings, the prevailing thought was that possession of the *MGMT* methylation gene predicted favorable response to treatment with temozolomide. This premise of using the *MGMT* methylation gene for prediction of response to treatment with temozolomide was challenged in 2011 by the RTOG 0525 study[14]. The purpose of this study was to look at a proposed strategy for overcoming acquired temozolomide resistance, specifically whether there was a survival difference between the use of standard schedule of temozolomide or an altered schedule in which the same total dose of temozolomide was delivered in higher fractions, allowing for a 3 wk on, 1 wk off dosing. When these patients were stratified based on MGMT status, there was an OS of 23.2 mo in patients with tumors possessing the *MGMT* methylation gene *vs* 16 mo in those harboring unmethylated MGMT status. Thusly, the current paradigm is that possession of the *MGMT* methylation gene prognostically bodes better for patients receiving standard adjuvant treatment than those that do not possess in the general population but does not necessarily predict response to treatment with temozolomide. An important demographic caveat exists, however. It was found on that basis of multiple studies that the *MGMT* methylation gene does predict favorable responses in terms of survival benefit in elderly patients (age greater than 70) with GBM who receive TMZ and radiation *vs* RT alone[15-17]. This is important in that uses of different modalities of treatment necessarily must be used more conservatively and sparingly in elderly patients who have more limited physiological reserve with which to contend with the ill effects of such treatments.

Interestingly, there is another more nuanced twist to the MGMT story. Recent studies have revealed that in some tumors, the *MGMT* gene-and resistance to TMZ--is effectively silenced even without possession of the *MGMT* methylation gene. What these studies have found is that *MGMT* expression is also post-transcriptionally regulated by micro-RNAs[18]. MicroRNAs (miRNAs) are non-encoding RNA molecules 20-23 nucleotides in length that inhibit the translation and stability of messenger RNA (mRNA). MicroRNAs have a potent presence in the regulation of post-transcriptional gene expression as they "flag" mRNAs which leads to their decay and influences essential cell functions, *i.e.*, replication, proliferation, metabolism, programmed cell death, *etc*.[19]. Low *MGMT* expression in promoter unmethylated tumors was found to be due in part to the expression of miR-181d, a miRNA that suppresses *MGMT* expression. There have been additional micro-RNAs identified that bind directly to the MGMT 3' UTR and purportedly result in loss of MGMT protein expression both in pre-clinical and clinical studies.

Deletion, mutation and LOH on chromosome 10

There are a number of genes on chromosome 10 of which mutation, deletion or LOH has been established in the development of GBM malignancy. These will be considered in turn.

A well-described phenomenon engendering tumorigenesis is LOH. In somatic cells, many tumor suppressor genes bear heterozygosity by merit of having inherited unique single nucleotide polymorphisms in different regions in that gene. Thusly, one allele in the pair for that gene is different from the other. In the process of LOH, a portion of or a complete chromosome in a diploid pair is deleted. If this portion contains a tumor suppressor gene, then the cell containing that deletion exhibits LOH for that gene or chromosome. When the remaining copy of the tumor suppressor gene incurs a mutation, the cell is no longer protected by that tumor suppressor gene and the biology of malignancy is begotten.

The LOH phenomenon specifically involving alleles of tumor suppressor genes in parts or all of chromosome 10q has reliably been demonstrated in the molecular pathogenesis of GBM[20]. A specific example is allelic deletion of the phosphatase and tensin homolog gene, or PTEN, located at locus 10q23[21] (Figure 2).

The wild type *PTEN* gene is a tumor suppressor. The product of this gene is involved in many different signaling pathways in its capacity as a phosphatase. The most important of these pathways is the PI3K/Akt pathway[21]. When an extracellular ligand binds to its correspondent receptor, *e.g.*, EGFR, HER2, IGFR, the protein PI3K is activated and creates PIP13. PIP13 in turn recruits the Akt to the intracellular surface of the cell membrane and subsequently activates the PI3K/Akt pathway. Activity is positively regulated by the PIP3 gene product. This pathway promotes a number of progrowth phenotypes, including cell cycle progression, protein synthesis, inhibition of apoptosis and cell migration. When PIP3 is dephosphorylated by PTEN to PIP2-a, the PI3K/Akt pathway is downregulated and antagonizes the progrowth phenotype. Thusly, when PTEN activity is lost through mutation or LOH, PIP3 accumulates and begets malignant growth though constitutive activation of the PI3K/Akt pathway. Mutations in *PTEN* have been implicated in a variety of malignancies, including prostate, gyn malignancies, breast, pancreatic, melanoma and GBM[21].

PTEN LOH mediating malignant features in GBM has been found to occur in as much as 60%-80% of all cases[21]. Historically, studies concerning LOH or mutation in *PTEN* had proposed a value in prognostication, *i.e.*, that loss of PTEN would make for a poorer prognosis[21]. This was particularly so prior to acceptance of TMZ as standard adjuvant treatment of GBM. However, a recent study out of Cedars-Sinai medical center appears to refute this understanding[22]. Indeed, the premise

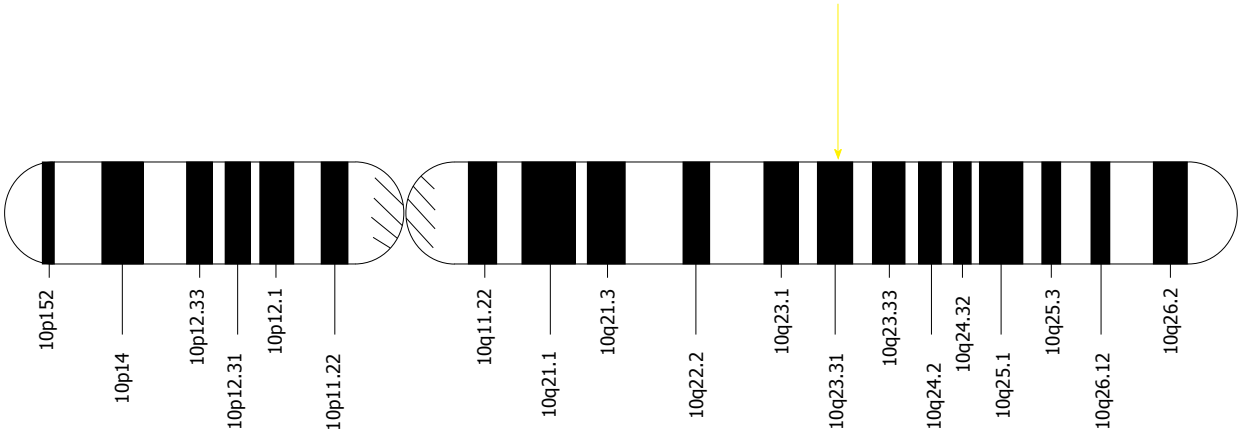

Figure 2 *PTEN* locus on chromosome 10. *PTEN*: Phosphatase and tensin homolog.

of the study was to update the understanding of the significance of this molecular marker in the current TMZ treatment era. In this study, the authors retrospectively looked at the presence or absence of *PTEN* in 155 tissue samples from patients who underwent craniotomy for resection of GBM between 2007 and 2010. The majority of these patients (80.7%) were treated with standard adjuvant radiation and TMZ chemotherapy after surgical tumor resection. What they found was that the loss of PTEN *via* LOH, mutation, or deletion was not associated independently with poorer prognosis as had been previously assumed. What they did find was that in their multivariate analysis, certain features assessed were significant predictors of worse prognosis; these included: older age ($\geqslant$ 65), poorer functional level based on KPS score, partial resection of tumor, and not instituting standard adjuvant therapy. Interestingly, the authors also found evidence that appeared to corroborate the supposition that GBM cells that had PTEN loss were more susceptible to TMZ treatment. This was suggested in an *in vitro* study that found that glioma cell lines lacking PTEN were more sensitive to TMZ treatment than PTEN possessing glioma cell lines. The thought process is that the lack of PTEN makes those cells less capable of repairing double-stranded DNA breaks effected by TMZ, and thus makes TMZ more chemotherapeutically effectual. What this implies is that the reason the authors found no statistically significant difference in patient outcome based upon PTEN loss or presence alone is because the increased effectiveness of TMZ in PTEN loss would effectively even out the outcomes between the two groups. This would appear to explain the difference found between this study and prior studies that did not evaluate patients who had undergone adjuvant TMZ treatment. Thusly, it is not entirely clear that *PTEN* loss is not an independently poor prognostic factor. Moreover, this seems to suggest that *PTEN* loss would predict a more favorable response to TMZ though the outcome would appear not to be substantively different from patients with the presence of *PTEN*.

LOH has been found to occur in increased incidence in other genes located on chromosome 10 in GBM patients. Summarily, these, alongside LOH of *PTEN* on chromosome 10q23, indicate that this phenomenon alongside mutations on chromosome 10 may possess prognostic value when found in patients with newly-diagnosed GBM. However, as discussed above, there remains more to be elucidated in the context of the contemporary treatment paradigm before such mutations may be reliably used for such prognostication purposes.

1p/19q codeletion

The next molecular phenomenon in GBM pathogenesis to be discussed is the 1p/19q codeletion. This results from an unbalanced translocation between chromosomes 1p and 19q and leads to LOH. This molecular signature has been found to have tremendous significance and clinical utility in the evolving paradigm of molecular based prognostication and treatment of high grade glioma.

There have been three randomized clinical trials that have investigated the 1p/19q codeletion in GBM and found that it actually confers a survival benefit to patients whose tumors possess this codeletion and are receiving RT and/or alkylating chemotherapy. These trials will be discussed in turn.

The first trial to be considered here is the RTOG 9402 phase Ⅲ randomized study, which included 289 patients with grade Ⅲ anaplastic oligodendroglioma or anaplastic oligoastrocytoma treated with either adjuvant RT alone or four cycles of chemotherapy (Procarbazine/CCNU/Vincristine) followed by RT (PCV - > RT)[23]. The primary endpoints were assessing differences in PFS and OS between the two arms in the study. In this, they found at 3 years out that there was a benefit in PFS in the PCV - > RT arm (2.6 years) over the RT alone arm (1.7 years); however, there was no significant difference at that time in OS (4.7 years in the RT arm *vs* 4.9 years in the PCV - > RT arm). The researchers also assayed tissue samples for the 1p/19q codeletion and assessed whether this had any bearing upon either PFS or OS. Of 201 patients assayed with fluorescence *in situ* hybridization (FISH), 93 (46%) were positive for

the codeletion. It was found that these patients had a survival benefit conferred by the codeletion over the wild type tumors. Irrespective of the treatment arm, those patients possessing the 1p/19q codeletion had a median OS of > 7 years whereas the median OS in patients without the codeletion was 2.8 years. Though treatment at this juncture did not appear to have any bearing on survival, extended follow up 2012 confirmed the better prognosis for the 1p/19q codeletion group and that PCV - > RT also appeared to improve survival over RT alone in those with the codeletion. The median OS in non-codeleted tumors was 2.6 years and 2.7 years in the PCV - > RT and RT alone group, respectively. However, patients with codeleted tumors had a median OS of 14.7 and 7.3 years in the PCV - > RT and RT alone groups respectively. Thusly, it appeared from this trial that the 1p/19q codeletion possessed both prognostic and predictive value.

Soon thereafter, the EORTC 26951 trial was conducted, compared RT alone to RT followed by six cycles of PCV (RT - > PCV) in 368 patients with anaplastic oligodendroglioma or anaplastic oligoastryocytoma randomized between the two arms[24]. Summarily, the outcome was analogous to the aforementioned trial on the primary endpoints of PFS and OS; PCV and sequential RT did increase PFS from 13.2 mo to 23 mo but had no bearing on OS (40.3 mo for PCV - > RT *vs* 30.6 mo for RT alone) at 60 mo out. The researchers in this study also used FISH to assay tissue for the 1p/19q codeletion; 78 patients (21%) were positive for the codeletion. As with the RTOG study, 1p/19q codeletion was prognostic and conferred a better outcome irrespective of therapeutic intervention. At 60 mo out, those patients possessing the 1p/19q codeletion did not reach a discrete median OS whereas those without the codeletion and treated with RT followed by PCV had a median OS of 25.2 and 21.4 mo for those treated with RT alone. Results after extended follow up of 12 years in 2012, again mirrored those of the RTOG trial at extended follow up, with RT - > PCV yielding a greater OS (no median OS reached in these patients) over RT alone (median OS of 9.3 years) in patients with the 1p/19q codeletion. This survival benefit was not seen in those patients without the codeletion; in this contingent, those receiving RT - > PCV had an OS of 25 mo and those receiving RT alone 21 mo.

A third trial known as NOA-04 conducted by the German Neuro-Oncology Group prospectively evaluated 318 patients with anaplastic astrocytoma, anaplastic oligodendroglioma, and mixed anaplastic oligoastrocytoma treated with RT, PCV, or TMZ-by the ratio of 2:1:1, respectively[25]. Patients who experienced excessive toxicity or progression after RT were then randomized to receive either PCV or TMZ, or patients with similar outcomes during or after primary treatment with chemotherapy were then administered RT. The primary endpoint was treatment failure, and 43% reached that endpoint at 54 mo out. On PFS and OS, the three groups were found to have similar results. In this study, FISH assays of tissue found 74 patients (23%) to possess the 1p/19q codeletion. When assessing these patients against wild type patients on the primary endpoint, there was, regardless of therapeutic intervention, an improvement of almost 50% in treatment failure. What should be understood, however, is that the benefits conferred by the codeletion had no bearing upon those patients with anaplastic astrocytoma. The aggregate of the studies described above show that the 1p/19q codeletion has both prognostic and predictive utility in malignant gliomas and may thereby represent a tool to guide clinicians in prognostication and treatment planning for patients with these malignancies.

IDH1/IDH2 mutations

The genes IDH1 and IDH2 are molecular markers that demonstrate prognostic value in patients with glioblastomas as well as lower grade gliomas. Isocitrate dehydrogenase (encoded by IDH1 in the cytoplasm and by IDH2 in the mitochondria) in its wild type form produces alpha-ketoglutarate[26]. Mutations in these genes encode an aberrant enzyme that turns alpha-ketoglutarate into an onco-metabolite, D-2 hydroxyglutarate. D-2 hydroxyglutarate controls the oncogenicity of IDH mutations. Based upon mutation status, gliomas may be classified as IDH-wild-type or IDH-mutant. IDH-wild-type gliomas include grade Ⅰ pilocytic astrocytomas and primary GBMs. Tumorigenesis in this case is, therefore, independent of the IDH status and is mediated by other oncogenes. IDH-mutant gliomas include grade ⅱ and grade Ⅲ gliomas as well as some secondary GBMs. What is interesting is that within a given histological class, IDH mutants carry a better prognosis than IDH wild types. For example, in WHO class Ⅳ tumors, secondary GBMs (IDH mutants) carry a better prognosis than primary GBMs (IDH wild types). An analysis of 382 high grade gliomas in 2010 found that IDH status has greater prognostic value than histological grade[27]. Thusly, it is now being realized that grouping gliomas by IDH status is more useful for prognostication than grouping by histological grade and morphology.

p53 mutations

It is well known, that protein p53, with a gene located on the short arm of chromosome 17 (17p13.1), is one of the main tumor suppressors. It is a transcription factor that activates the expression of genes that will induce the G1 cell cycle arrest in response to cell stress and DNA damage. Hence, the somatic and the germline mutations of p53 are associated with a variety of human cancers[28,29].

The Cancer Genome Atlas Network (TCGA) reported p53 mutations in 37.5% of the newly diagnosed, and in 58% of the previously treated GBM samples[30]. As far as the pathogenesis of malignant gliomas is concerned, the mutations in p53 and its regulatory pathways primarily play a role in development of secondary gliomas as

opposed to the primary glioblastomas[31-33].

Other than the cell cycle progression, several cellular processes are thought to be affected by p53 such as: the response to DNA damage, apoptosis, and the cellular differentiation and neovascularization[34]. Based upon cellular homeostasis, the p53 gene product is typically low in normal cells and increased in cells affected by DNA damage, where it exhibits a relatively short half life, being degraded by the Murine Double Minute 2 (MDM2) protein in the cytoplasmic milieu[35].

Apart from the events that mutate the p53 itself, the mutation of genes encoding its functional regulation are found in approximately 70% of GBM samples, mainly ARF, 55%, MDM2, 11%, and MDM4, 4%[36]. The MDM2 and MDM4 proteins that function as E-3 ubiquitin ligase, degrade the p53 and repress its function. It has been confirmed, that the amplification of MDM2/MDM4 proteins inactivates the transcriptional activity of p53, resulting in abrogation of its antiproliferative and apoptotic effects[37,38].

The CDKN2A locus has been shown to present another frequent mutation in glioblastomas. In addition to encoding the p16INK4a, that is a specific inhibitor of CDK4/6, this locus encodes a second protein, the p14ARF, whose expression also induces a cell cycle arrest. The p14ARF acts by binding MDM2, thus promoting its rapid destruction, and leading to the stabilization and accumulation of p53. For INK4a/ARF locus mutation, the protective, antitumorogenic role of the p14ARF is lost, due to the suppression of p53[39]. The importance of this locus is also confirmed by observation, that in mice models the homozygous deletions of both p16 and p14, are correlated with the increased progression from lower to higher grade gliomas and poorer survival rate in patients older than 50 years[40].

Molecular profiles in GBM

In 2010, the group of Verhaak *et al*[41] published results of a study in which they utilized the genomic sequences of 91 GBM patient made available by TCGA Research Center to look at patterns of gene mutations and expression in different tumors that may allow for categorization of these tumors into distinct subclasses. They found they were able to find distinct genomic patterns that hewed to a classification system that would allow them to classify any given tumor into one of four subtypes: Proneural (PNL), Neural, Classical (CL) and Mesenchymal. In addition to allowing for the distinct biology for tumors of each of these classes to be contrasted with the others, it was posited that this may have utility for prognostication and/or predicting response to treatment.

As discussed, GBM by histology is characterized as one entity. This has been found to be a considerable oversimplification that does not account for the differences in biology between different GBM tumors. Genetics have revealed that there exist multiple subtypes of GBM.

What is clinically significant here is that the biology of each subtype confers upon it differences in prognosis and/or response to treatment from the other subtypes. As to the final consensus on how many genetics-based subtypes there actually are, this remains to be determined and studies are ongoing to this end.

One such study that provided a compelling insight into what is likely to be representative of the future of GBM classification came from a group out of Belgium in 2012[42]. The authors predicated their investigation upon the results of Verhaak *et al*[41] cited above, namely exploiting unique patterns of genetic mutations to classify GBM into distinct biological subtypes that each have unique clinical characteristics. The goal of the study was broadly twofold: to devise a relatively simple assay of mutations in tumor samples for classification into one of two subgroups; and to try to ascertain biological features of tumors from each subgroup that have demonstrable value in making clinical inferences. To this end, the authors did a retrospective analysis of 100 patients with new, treatment naive GBM. They utilized immunohistochemistry (IHC) to quantitatively assay tumor samples from these patients for the presence or absence of mutations in 3 well-characterized genes in GBM-EGFR, PDGFRA, and p53. Based upon the pattern of presence or absence of mutations in these genes, the investigators were able to discern two subtypes of GBM: the CL subtype and the PNL subtype. To be sure, these subtypes had been initially described by Verhaak *et al*[38] but the association with the IHC mutational analysis done here was entirely new. The CL subtype is characterized by positive immunostaining for EGFR and is negative for p53 and PDGFRA mutations. The PNL subtype, on the other hand, is EGFR negative and demonstrates positive immunostaining for p53 and/or PDGFRA. Of the initial cohort of 100 GBM specimens, 93 were able to be quantitatively assessed for these genetic signatures. Based upon the criteria set outlined, 35 specimens were found to belong to the CL subtype and 56 were found to belong to the PNL subtype; the other 2 specimens did not stain for any of the three markers. The endpoints assessed for the patients in this retrospective analysis were PFS and OS. The former was defined as the time elapsed from the date on which the tumor was resected to the date on which the tumor was found to have recurred or if the patient died from recurrence of tumor. The latter was defined as the time elapsed from the date on which the tumor was resected to the date the patient died due to tumor progression. Summarily, the study found the following of notable clinical significance. Firstly, patients with tumors of the PNL subtype had a statistically significantly higher median OS of 10.5 mo than the median OS of 5 mo for patients of the CL subtype (P = 0.047). Similarly, a mortality risk reduction of 52% was linked to the PNL subtype when compared to the CL subtype. Hence, it was suggested that the delineation of a given GBM patient to one of the two subtypes would possess value in prognostication. Furthermore and not insignificantly, the authors demonstrated that the information needed

to make this categorization, *i.e.*, PDGFRA, EGFR, and p53 status, is relatively easily obtainable through IHC staining. Secondly, the authors found that these two subtypes possess biological characteristics conferred by their respective genetic signatures that make their response to adjuvant adjuvant chemotherapy different from one another.

Specifically, they found that temozolomide chemotherapy with radiotherapy did dramatically improve survival of patients of the CL subtype. This was not the case in that contingent of CL patients receiving radiotherapy alone, who showed no significant improvement in OS compared to patients receiving no treatment or palliative management. Interestingly, treatment modalities had quite the opposite effect on patients of the PNL subytype. These patients who received radiotherapy alone saw a significant improvement in OS over those who received no treatment or palliative management. However, the addition of temozolomide to radiotherapy did not improve survival in this subset of patients as it did in the CL patients.

Autophagy

When considering the multimodal actions of TMZ as a chemotherapy agent and contextualizing this in the problem of chemotherapy resistance, a topic of recent research interest is autophagy.

Autophagy, known as type Ⅱ programmed cell death, is a catabolic process during which cells self-digest intracellular organelles. When allowed to go to completion, autophagy results in cell death[43,44]. Biologically, it serves two functions: as an intracellular mechanism of disposing of damaged organelles and proteins, and for catabolism of substrates during cellular stress in order to generate energy needed for cell survival. As may be intuited, persistent autophagy does in many cases result in cancer cell death. However, there is also mounting evidence that autophagy may also drive the damage response that cancer cells use to avoid death when exposed to metabolic and therapeutic stresses.

Knizhnik *et al*[45] demonstrated in glioma cells that TMZ can induce cell death *via* a complex process between apoptosis, autophagy, and senescence. Senescence represents a state when viable cells stop synthesizing DNA with the unknown endpoint of either survival or death. They demonstrated that TMZ - induced cell death could be accomplished by two mutually exclusive pathways: by apoptosis alone (*via* the caspase-mediated pathway) or by autophagy followed by cellular senescence. It was found that the autophagy pathway inhibited the appositional apoptosis pathway, and the cells progressed to senescence. Thus, it is proposed that autophagy may be a survival mechanism whereby gliomas undergo senescence rather than immediate death *via* apoptosis when therapeutic doses of TMZ are used. Knizhnik *et al*[45] also found that autophagy, senescence, and apoptosis of glioma cells occurred at 72, 96, and 120 h after TMZ exposure, respectively.

As a result of TMZ-induced autophagy, it is possible that the high recurrence rate in glioblastoma patients and the unsatisfactory clinical survival rate might not only be due to the resistant mechanisms of tumors such as MGMT and deficiency of MMR but also due to autophagy allowing the tumor to survive where it should otherwise undergo apoptosis. As such, investigations are underway to see if adjunctive treatment with an autophagy inhibitor may enhance the beneficial therapeutic effects of TMZ for patients with GBM.

Vascular endothelial growth factor

GBM is one of the most vascularized human tumors and, alongside high expression of various proangiogenic factors, vascular proliferation is one of its defining pathologic features[2]. GBM cells produce proangiogenic factors; one of, if not the, best characterized of these is vascular endothelial growth factor (VEGF).

VEGF consists of a family of 5 glycoproteins: VEGF-A, VEGF-B, VEGF-C, VEGF-D, and placenta growth factor. These factors bind with their corresponding tyrosine kinase receptor (VEGFR-1, VEGFR-2, and VEGFR-3) and activate a signal transduction cascade that results in the development of angiogenesis, increased vascular permeability, and lymphangiogenesis. Of these, VEGF-A plays the greatest role in tumor angiogenesis along with tumor cell proliferation and migration. Thusly, elevated levels of VEGF-A in patients with cancer--specifically that of breast, lung, colon, uterus, and ovary--confers a graver prognosis[46].

Bevacizumab is a humanized monoclonal antibody to VEGF-A[47]. This antibody prevents the interaction of VEGF with target receptors VEGFR-1 and VEFGR-2 on the surface of endothelial cells. This in turn prevents downstream signaling that would normally induce tyrosine phosphorylation and the subsequent cascade of signal transduction events that would lead to endothelial cell survival, proliferation and vascular permeability. The composite effect of causing regression of existing microvasculature, inhibition of new vessel growth and normalization of the surviving vasculature (which leads to reduced vascular permeability and reverses peritumoral edema) bears a particularly germane pertinence to GBM. In the United States, bevacizumab has been approved for recurrent GBM based on studies that showed improvement in PFS but not OS[47].

In February 2014, Chinot *et al*[48] published randomized, double-blinded, placebo controlled trial on newly diagnosed GBM patients where they compared standard radiotherapy and TMZ for newly diagnosed GBM with or without bevacizumab. The study met the first primary endpoint of improved median PFS with statistical significance ($P < 0.0001$), finding a 4.4 mo improvement in median PFS of the experimental group (10.6 mo) over the control group (6.2 mo).

The OS at 1 year ($P = 0.049$) was 72.4% and 66.3% in the experimental and control groups, respectively. At 2 years ($P = 0.24$), the OS was found to be 33.9% and 30.1%, respectively, which was

not statistically significant. The experimental group receiving Bevacuzimab maintained a longer quality of life and performance status and required less steroids. However, the study noted that there was a clearly greater number of clinically significant adverse events in the Bevacizumab group than the control group.

The RTOG 0825 study, published in the same month as the study by Olivier *et al*[49], came to a similar conclusion: namely that adding bevacizumab to standard of care RT/TMZ provided discernible benefits for PFS but not for OS. Notable adverse effects in the bevacizumab group were hypertension, thromboembolic events and intestinal perforation, consistent with previously reported side effects of this medication.

The BELOB trial by Taal *et al*[50] out of Europe investigated three lines of therapy for patients with recurrent GBM: single-agent bevacizumab, single-agent lomustine and combination therapy with bevacuzimab plus lomustine. Results demonstrated 9-mo OS to be 43% in the lomustine group, 38% in the bevacizumab group and 59% in the combination group. Extrapolating from these results, the authors strongly questioned the role for single agent bevacizumab in recurrent glioblastoma. However, it provided a compelling indication for further investigations of combination bevacuzimab with lomustine, particularly in a phase Ⅲ trial.

The aggregate of data from clinical trials on bevacuzimab for newly-diagnosed and recurrent GBM reveals that the proposed mechanism of action of bevacuzimab in antagonizing the VEGF pathway is not enough on its own to explain the observed results. It has led investigators and the scientific community to realize that there are much more complex regulatory mechanisms in angiogenesis at work than previously recognized.

Hypoxia and treatment resistance

Recent evidence has indicated that prolonged anti-angiogenic treatment leads to development of progressive hypoxia in tumor tissues which in turn has led to the recognition of an entirely novel paradigm of treatment resistance. VEGF blockade of its own causes only a small reduction in tumor burden but does induce a strong depletion of large and intermediate-sized blood vessels with a subsequent reduction in vascular leakage and intratumoral blood flow.

This result in a hypoxic microenvironment within the tumor which is proposed to provoke significant tumor cell invasion.

Hypoxia-inducible factor-1 (HIF-1) is a transcriptional complex belonging to a family of transcriptional factors known as hypoxia inducible factors (HIFs) that is activated in response to hypoxia and growth factors. HIFs are heterodimers composed of an oxygen-sensitive HIF-alpha subunit and a HIF-beta subunit. Under normal homeostatic cellular conditions, HIF-alpha binds to the tumor suppressor protein von Hipel-Landau (vHL), which leads to degradation of HIF-alpha.

However, under conditions of hypoxia, there is an abrogation of the interaction between HIF-alpha and vHL as a result of which HIF-alpha gets stabilized. This leads to dimerization of HIF-alpha which then allows it to bind to hypoxia responsive elements on promoters of genes inolved in promoting cell survival, motility and metabolism. The activation of HIFα also plays a regulatory role in the expression of VEGF and inducible nitric oxide synthetase facilitating angiogenesis and the tumors cell's access to the circulatory system. Two HIFα subunits, HIF-1α and HIF-2α are primarily responsible for regulating tumors adaptation to hypoxia. HIF-1α is widely expressed in several tissues, while HIF-2α has a more restricted expression pattern and is associated with cancer initiation or tumor progression. Thusly, HIF-1 plays a central role in tumor progression, invasion, and metastasis. Indeed, overexpression of the HIF-1α subunit has been observed in many human cancers and is associated with a poor prognostic outcome with conventional treatments[51].

Preclinical trials of recent have revealed some very intriguing characteristics of tumor vasculature. Three major mechanisms have been proposed for the development of new tumor vasculature: proliferation from preexisting vessels, colonization by circulating endothelial cells or colonization by proangiogenic bone marrow cells. This last phenomenon has come to be denoted as vasculogenesis[52].

In specific, vasculogenesis itself depend on three major pathways: (1) mobilization and recruitment of proangiogenic bone marrow derived cells (BMDCs) into tumor milieu; (2) retention of these BMDCs in hypoxic tumor tissues; and (3) vascularization dependent on $CD11b^+$ myelomonocytic cells.

Hypoxia leads to induction of the transcription factor HIF-1 which has been shown to be a major recruiter of BMDCs to tumors including GBM. Retention of these cells is dependent on secretion of stromal cell derived factor-1 (SDF-1, CXCL12) which binds its receptor, CXCR4, on the BMDCs. Thus has been elucidated the link between hypoxia in GBM and vasculogenesis.

This in turn has led researchers to propose the means by which bevacuzimab engenders treatment resistance.

The proposed hypoxia model as discussed has been further supported by studies looking into inhibitors of the modulators of vasculogenesis. In xenograft models, the HIF-1 inhibitor NSC-134754 and AMD3100, an inhibitor of the SDF-1/CXCR4 interaction, compellingly found little to absent tumor regrowth following irradiation[53].

Hypoxia has been also been proposed as a means of activating autophagy, the lysosomal degradation pathway that, as discussed earlier, likely promotes tumor cell survival[54]. The mechanisms by which hypoxia induces autophagy need elucidation, but the finding that BNIP3, a downstream target of HIF-1α, is essential to hypoxia-induced autophagy suggests a likely mechanism.

Cancer stem cells

An additional important developing point of interest with therapeutic potential is the identification of cancer cells with stem cell-like properties. It has been hypothesized that a subset of cells known as the cancer stem cells exist within a tumor with stem cell like properties and can initiate primary tumors as well as recurrences by way of their self-renewal capacity and inherent resistance to therapeutics.

Glioblastoma contains multipotent tumor stem cells (GSCs) that could be responsible for populating and repopulating tumors.

Specific criteria are required to define GSCs: (1) the ability to self renew; (2) the ability to differentiate into different lineages (multipotency); and (3) the ability to initiate tumors in animal models which recapitulate the original disease phenotype and heterogenicity[55,56].

Multipotent neural stem cells have the ability to differentiate into neurons and glia (astrocytes and oligodendrocytes). Physiologically, stem cells have a long life expectancy and divide frequently which makes them more susceptible for tumorigenesis.

The process of neurogenesis occurs in two major regions of the adult brain: the subventricular zone of the lateral ventricles (SVZ) and the subgranular layer of the hippocampal dentate gyrus[57].

Neuronal stem cells (NSCs) are regulated by the orchestration of intrinsic factors with extrinsic signals from surrounding microenvironment, defined as the neurogenic niche. A niche represents a specialized anatomic compartment formed by cellular and acellular components that integrates local and systemic factors, supports maintenance and survival and actively regulates the function and proliferation of NSCs.

It has been hypothesized that once neurogenic niches house NSCs (which have a relatively large chance of becoming cancerous cells) and support maintenance, survival and proliferation, they become vulnerable sites for growth and proliferation of transformed cells. It is believed that the SVZ gives rise to the highest number of glioblastomas and this has led to efforts looking at this cell population as a potential therapeutic target.

The sole process of neurogenesis depends on a complex cascade of molecular signaling pathways. These candidate pathways include Notch[58], bone morphogenic protein[59], Wnt[60] and sonic hedgehog (Shh)[61].

Blockage of Notch signaling with γ-secretase inhibition, inhibits self-renewal, and causes CD133+ cell depletion[62]. Transforming growth factor-β (TGF-β) signaling promotes GSC self-renewal[63]. Shh signaling (important during embryonic development) plays an important role in GSC maintenance by promoting self-renewal and expression of stem cell genes[64], whereas blockage leads to apoptosis, delay in tumorigenesis and inhibition of GSC self-renewal and migration[65].

Similarly to that of normal stem cells, GSCs are found in a microenvironment that provides ideal condition for tumor maintenance. The tumor perivascular niche is composed of heterogeneous cell groups, including astrocytes, endothelial cells, macrophages, microglia, non-tumor initiating cells, and, indeed, tumor stem-like cells[66].

GSC chemotherapy resistance and radiotherapy resistance

Multiple mechanisms leading GSCs to chemo-resistance have been identified in pre-clinical studies. These include: increased activity of ABC-type transporters present on the cell surface that extrude chemotherapeutic agents to the extracellular space[67]. These chemo-resistant cells have been identified in GBM cells *via* flow cytometry with a specific pattern of expression of surface antigens (CD133+, CD117+, CD90+, CD71+, CD45+)[68]. Further corroborating the important role GSCs have in chemo-resistance, CD133 is highly expressed in recurrent tumors and transcriptional analysis of these cells demonstrates concurrent over-expression of anti-apoptotic genes[69]. Parada *et al*[70] applied these findings and showed that a restricted Nestin+ GSC population could regenerate tumors after being treated with temozolomide. Others have attempted selective ablation of this cell population and this only led to tumor growth arrest, supporting the hypothesis that GSCs resist current standard chemotherapy and have intrinsic properties of chemo-resistance. In addition to the above, GSCs have slow cell cycles, generally quiescent and are immune to exposure to chemotherapy because these traditionally target actively cycling cells. GSCs also have the ability to evade irradiation with the development of clones that over-express GSC markers as well as triggering over-activation of the Notch and TGF-β signaling pathways[71,72].

CONCLUSION

GBM has historically been and indeed remains a formidable challenge for clinicians and has maintained a grim prognosis not much changed from the very inception of conventional treatment. This is despite a profusion of significant recent discoveries regarding its unique biology and intricate molecular pathogenesis. However, with the elucidation of these recent and ongoing findings, there are a number of exciting studies underway investigating entirely novel treatment modalities that exploit these recent revelations. It is expected that with fruition of validated results in animal models and progression to phase Ⅲ clinical trials, a veritable revolution will take place in both the diagnosis and treatment of this most malignant of primary brain cancers.

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