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ORIGINAL ARTICLE

Mutations of the *human interferon alpha-2b* gene in brain tumor patients exposed to different environmental conditions

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This study was aimed at finding out mutations of an anticancerous, antiviral and immunomodulatory gene (*human interferon alpha-2b*) in low- and high-grade brain tumor patients and correlate from hematological profiles. A molecular analysis was performed in which DNAs were extracted from brain biopsy samples of brain tumor patients. The gene was amplified through the PCR technique, and genetic data from sequencing were analyzed by bioinformatics to determine how mutations will lead to changes in human interferon alpha 2b protein in patients. A total of 38% gene mutations were identified among brain tumor patients. The highest percentage of (36%) frameshift mutations was identified. Hematological analysis shows modulations in the 'lymphocytes' parameter in a majority (64%) of the brain tumor patients. Environmental factors have been reported as risks of brain cancer. Patients were found to be under environmental stress from contaminated drinking water and from local gamma radiations. Brain tumor patients were found to have various mutations in an immunomodulating *human interferon alpha-2b* gene. These patients had immunosuppression that was further affirmed from their hematological profiles. This analysis may be helpful to develop certain biomarkers that may be used to develop novel immunotherapeutical drugs, which enhance a better immune response.

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INTRODUCTION

This study was aimed at finding out mutation(s) in an immune response gene (that is, *human interferon alpha-2b*) in pathologically confirmed low-grade and high-grade brain tumor patients after surgical excision. These patients were not treated with any chemotherapeutical or radiotherapeutical treatments when sampling was conducted. Brain tumor patients were included from an outpatient neurosurgery clinic during the years 2013 and 2014. It is evident that environmental factors influence human health, and most importantly some types of exposures affect human genes. These patients were found to be under some environmental stresses, as some of them belonged to those areas in which natural terrestrial radioactivity is present owing to uranium deposits and, of course, their houses were made of radioactive-contaminated materials. Therefore, they were found to be exposed to radon levels as well. These patients were taking food and drinking water which was from radioactive contaminated agriculture from uranium emitting rocks and soils. Such dwellers were also exposed to natural radioactivity because of their occupations, as they were working on the contaminated agricultural fields where radionuclides are found. Ionizing radiations (IRs) such as x-rays or gamma rays from the natural environment have long been studied and associated with radiation-induced cancers, as they can cause genetic damage. The other environmental stress was found in terms of unsafe drinking water. Many patients were not having safe (filtered) water probably owing to the presence of nitrate/nitrite contamination from agricultural activities or chlorinated by-products present in city water supply. These environmental stresses were identified on the basis of the survey analysis from the patients included in this study. Attempt has been made in this study to not only provide comprehensive molecular analysis related to mutations in *human interferon alpha-2b* gene in brain tumor patients but also correlate the findings related to immune response of the patients assessed from their hematological profiles. This study also provides a short review related to known and suspected neurocarcinogens, previous molecular analyses on brain tumors and introduction to interferons and the immune response.

Brain tumor incidence and grading

Brain tumor develops because of cellular genetic alterations that allowed them to escape from normal regulatory mechanisms and destruction by the immune system. These alterations happen owing to different chemical/physical/biological neurocarcinogens.[1] Brain cancer is a distinct set of intracranial and heterogeneous group of tumors. Half of all primary brain tumors arise from cells within the brain, whereas others originate in the meninges or nerves. Brain cancer occurrences and rates of mortality have been increasing for the past 25 years.[2,3] Primary brain tumors account for $<2\%$ of all the human cancers, but brain cancer is the leading cause of cancer-related deaths in individuals <35 years. Further, this is the second most leading form of tumors in children as well. There is a poor survival rate observed for brain tumor patients, and still no any significant improvement is seen.[4] The worldwide incidence of primary malignant brain and central nervous system (CNS) tumors prevail in the world population as 3.6 and 2.5/1 00 000 persons per year among men and women, respectively.[5] Higher-grade brain tumors

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(WHO grades III and IV) without any treatment follow a more aggressive clinical course as compared with lower-grade tumors (WHO grades I and II).[6–8]

A majority of primary intrinsic tumors arise from glial cells, which are called gliomas, and according to Central Brain Tumor Registry of The United States (CBTRUS) 2005–2006 they represent ~40% of this type. They surround and support neurons, including astrocytoma, glioblastoma, oligodendroglioma, oligoastrocytoma and ependymoma.[2,8,9] Some gliomas grow slowly and incline to show a cure, whereas other gliomas are found to be growing fast, invasive, difficult to treat and are likely to reappear. Major symptoms of glioma include headaches, numbness, weakness, confusion and seizures. Grade I brain tumors are pilocytic astrocytoma, which mostly occurs in children in the cerebellum and occasionally in the cerebral hemispheres. This grade of brain tumors can occur in adults. Grade I brain tumors are slow growing and relatively benign. Grade II brain tumors are low-grade gliomas, which include astrocytoma, oligodendroglioma and mixed oligoastrocytoma. Grade II gliomas mostly occur in adults (20–50 years old) and are mostly found in the cerebral hemispheres. These tumors can recur because of their infiltrative nature. Grade II astrocytomas arise from astrocytes, and they constitute 15–25(%) of all gliomas.[8] Some grade II gliomas can recur and transform into more aggressive tumors (grade III/IV). Grade III tumors are inclined to grow faster and aggressively than grade II astrocytomas. Grade IV brain tumor includes glioblastoma multiforme (GBM), which is the most common primary malignant brain tumor, and it is the most aggressive form of cancer among all cancer types. GBMs are found to spread quickly, and they can easily invade other parts of the brain.[2,3,10–12]

The most common forms of benign nervous system tumors are meningiomas and schwannomas. Meningiomas account for up to 37% for all primary brain tumors.[13] Meningiomas initiate in the meninges, which is the layer of the tissue that surrounds the outer part of the brain and spinal cord. Meningiomas are the most common brain tumors in adults. Sometimes meningiomas run in families—for example, in those with neurofibromatosis syndrome. Grade I includes benign tumors, and their cells mostly appear like normal cells, and 80% of them are meningiomas. These tumors can be cured from surgery; however, some of them can grow very close to important structures in the brain or cranial nerves. Grade II includes invasive meningiomas, and their cells usually look slightly abnormal. Approximately 15–20(%) of meningiomas are grade II. Grade III includes anaplastic meningioma cells that appear to be the most abnormal. They make up only ~1–3(%) of the meningiomas. They incline to grow quickly, they can grow into neighboring brain tissue and bone and they are most likely to recur even after treatment, and some of them may even spread to various other parts of the body.[3,11,14] Diffuse astrocytoma (WHO Grade II, low-grade astrocytoma) arises from astrocytes and constitutes 15–25% of all gliomas. Although they are histologically low grade, these tumors almost always acquire the clinical distinguished characteristic of more malignant astrocytomas 4–6 years after the diagnosis, and they are mostly found to be destructive. The average survival range is between 2 and 10 years or more.[8]

Miscellaneous environmental risk factors for brain tumor incidence

It is reported in many studies that environmental factors and related occupational exposures have been associated with primary or malignant brain tumor incidence. There is also a concern that people involved in certain occupations, such as electrical, petrochemical, agriculture, nuclear, airline and hospital radiation department, may be at an elevated risk of brain cancer.[15–20] Several environmental and dietary neurocarcinogens have been reported to be associated with glioma and meningioma risks, but research is still underway to confirm such factors. Besides genetic disorders, head injury and trauma have been identified as risk factors. Exposure to vinyl chlorides, formaldehyde, pesticides, herbicides and insecticides, N-nitroso compound intake, dietary antioxidant intake, dietary calcium intake, dietary maternal N-nitroso compound intake, dietary maternal and early-life antioxidant intake, maternal folate supplementation, tobacco smoking, alcohol consumption and exposure to electromagnetic fields (EMF), volatile organic and nonorganic compounds, infectious agents, organic solvents, lubricating oils, acrylonitrile, polycyclic aromatic hydrocarbons, phenols and phenolic compounds, by-products of synthetic processed rubber, coal tars, carbon tetrachloride and carbon disulfide and so on might contribute to the increased risk for the formation of brain tumors.[12,16,18,21–23] It has been long suspected that contamination by nitrates/nitrites or by chlorinated by-products of sewage treatment for drinking water is associated with brain tumor formation.[7] Several familial cancer syndromes such as neurofibromatosis, tuberous sclerosis and Li-Fraumeni syndrome may also be associated with increased risk.[23–25]

Ionizing radiations (IR) and risk of brain cancer incidence

There are many known and suspected factors that are neurocarcinogens and are risk factors for brain cancer, and they are listed in various studies. IR is the only well-established risk factor for brain tumor incidence. According to[26], natural radon makes the highest percentage (43%) of all types of radiation exposures. Other radiation exposures are as follows: natural external radiations (18%), natural cosmic (14%), medical radiations (14%) and natural internal radiations (14%); miscellaneous artificial sources (for example, fall out, consumer products, occupational exposures and contaminants from nuclear industries and so on) constitute $<1\%$ of all the exposures. A relationship between meningioma risk and exposure to IR is well established, and this led scientists to examine whether variants in DNA repair genes contribute to influence this disease.[13,23,24,27–29] Therapeutic X-irradiation and frequent CT scans are also associated with an increased risk of brain tumors, and they are studied by many researchers[3,16,17,22,27,29]. It is known that gamma radiations induce mutagen sensitivity, and this is an important risk factor for brain tumor occurrence.[30] Inefficiency in DNA repair mechanisms induces chromosome instability syndromes, which are influenced by radiations.[18] The major mechanism triggers in the brain which is influenced by radiations and this is responsible for the structural alterations and DNA damage followed by pro-oxidant, pro-inflammatory and increased apoptotic response.[1]

Exposures from residential radon are responsible for most of the exposure to radiation in the population, especially indoors. A recent study found significant associations between long-term radon exposures in general populations and the risk of primary brain tumors. Exposure to radon and alpha emitters are reported from polonium-218 and polonium-214, which has been classified as a human carcinogen. Radon-222 gas arises from the radioactive decay of radium-226, which is present throughout the crust of the earth and in, of course, various building materials.[31] People are exposed to radon (highest percentage of exposure is 43%) because the building materials for their houses have radioactive elements mixed in them. Many epidemiological studies have investigated radon, which results from the decay of uranium-238, for the health of the population.[32] Terrestrial radionuclides such as uranium, thorium, radium-226, radium-228 and potassium-40 are found in certain geological areas and eventually nearby rocks and soil contaminate agricultural fields.[33] IR is known to induce a broad range of potentially mutagenic lesions in DNA, ranging from damaged DNA bases to DNA breaks. Radiation-induced somatic mutations at a number of loci have provided evidence of

the induction of point mutations in a single gene and of small and large deletions that may encompass a number of physically linked genes.[34] Although primarily lungs are affected because of radon inhalation, however dose calculations also predicted that inhaled radon and its progeny can cross the blood–brain barrier (BBB) and a relationship between residential radon and brain tumors may exist. Few studies suggested a statistically significant risk for brain cancer mortality for uranium miners as well. A study of uranium miners mentioned deaths due to brain tumors from radon exposure. A significant association is found between long-term exposure to residential radon in a Danish population and primary brain tumor risk.[35–38] Several studies have suggested that chronic exposure to low-dose radiation poses a genotoxic effect on somatic DNA of professionally exposed workers, such as workers exposed to hospital radiation from diagnostic or therapy machines. Some studies included interventional cardiologists who work in cardiac catheterization laboratories and are exposed to low doses of ionizing radiation. Therefore, long-term damage caused by IR may not only induce cancers but also various degenerative diseases. Damage to DNA is considered to be the main initiating event by which radiation damage to cells results in development of cancer.[39–41]

Natural radionuclides

In this study, 32% of patients belonged to Siwalik belt areas of Pakistan where natural radionuclides are present. The fluvial Siwalik Group of Pliocene to Pleistocene age was deposited in a foreland deep in front of the rising Himalaya.[42–44] The Siwalik Belt is 50–150 km wide and 1500 km long, passing through Southern Azad Jammu and Kashmir, Potwar, Southern Khyber Pakhtoonkhaw and Baluchistan. The Siwalik group was folded and faulted during Himalayan origin. The mineralization in the Siwalik group of rocks extends over a strike length of more than 1000 km.[45] This fluvial package deposited by a braided river system has volcanic ash intervals, which are a source of uranium mineral deposits and a very large number of uranium anomalies. Uranium deposits in the Sulaiman Range have been studied in Pakistan by Moghal.[46] The sandstone-hosted Siwalik deposits have been discussed by Basham and Rice.[47] The uranium mineral deposits occur in Baghal Chur (Dera Ghazi Khan),[42,48] Nangar Nai (Dera Ghazi Khan), Taunsa (Dera Ghazi Khan), Mochi Mar (Isa Khel) and Shavah-Shanawah (Kohat). Minor uranium occurrences are reported from various parts of Pakistan, including Kirthar Range, Sindh Province (South Pakistan), Kallar Kahar and Salt Range (Central-North Pakistan).[45,49,50] There are a large number of areas within the Siwalik Belt with numerous radioactive anomalies. In mineralized and some radioactive anomalous areas, uranium and its daughter isotopes are present in the ground water, soil and food chains. Moreover, respective residential areas have been found with elevated radon levels. Radiation-related ailments are often reported from the anomalous areas. The Higher and Lesser Himalayan igneous–metamorphic rocks in Pakistan have been discussed by Chaudhry and Ghazanfar.[51] The second group of uranium anomalies is associated with S-type granites and granite gneisses of Lesser Himalayan igneous–metamorphic belt, as well as Higher Himalaya.[51]

Molecular analyses regarding brain tumors

Significant advances have been made in molecular biology in order to understand genetic alterations linked to brain tumors. Brain tumors are classified on the basis of molecular alterations in genes, such as classical (*EGFR, PTEN, CDKN2A*), proneural (*PDGF, IDH-1, p53, PTEN, CDKN2A*), neural (*EGFR, p53, PTEN, CDKN2A*) and mesenchymal (*NF1, p53, PTEN, CDKN2A*).[52] The genetic alteration in the *LOH2* (loss of heterozygosity 2) gene located at chromosome 10q23 has been identified in brain, breast, kidney and prostate cancer cell lines.[53,54] Extensive further studies on candidate tumor suppressor gene *PTEN* (phosphatase and tensin homolog) or *MMAC1* (mutated in multiple advanced cancers 1) were conducted using different cancer cell lines.[55] The most common chromosomal abnormalities are found on chromosomes 1p, 7, 8q, 9p, 10, 12q, 13q, 19q, 20 and 22q, which are also linked to altered signaling pathways.[52] Previous molecular studies have identified important genetic events in human glioblastomas, including dysregulation of growth factor signaling *via* amplification and mutational activation of receptor tyrosine kinase (*RTK*) genes, activation of the phosphatidylinositol-3-OH kinase (*PI(3)K)* pathway and inactivation of the *p53* and retinoblastoma tumor suppressor pathways.[56] The genetic alterations found in GBM are complex and are mostly sporadic. Moreover, a small number of familial gliomas are associated with germline mutations— i.e., neurofibromatosis I and II, tuberous sclerosis complex, von Hippel–Lindau disease, Cowden disease, Li-Fraumeni cancer syndrome, Turcot syndrome and Gorlin's syndrome. Genes mutated in GBM include *EGFR*, *p53*, *p16INK4a/ p14ARF, PTEN* and *IDH-1*.[52]

The *PTEN* (phosphatase and tensin) gene, located on 10q23, has been implicated as a candidate tumor suppressor gene in brain, breast and prostate tumors. A study of different types of glioma patients analyzed for *PTEN* mutations suggested that *PTEN* gene mutations are restricted to high-grade gliomas.[57] A study highlighted the possible role of *S6K1* (ribosomal S6 kinase 1) in brain tumor progression and to predict patients' survival.[9] The *MDM2* (murine double minute 2) gene has recently been shown to code for a cellular protein that can complex the *p53* tumor suppressor gene product and inhibit its function. Some studies included several primary brain tumors and reported that the *MDM2* gene is amplified and overexpressed in 8–10% of glioblastomas and anaplastic astrocytomas. The *MDM2* gene also represents the second most frequently amplified gene after the epidermal growth factor receptor (*EGFR*) gene in these tumor types.[6,58] An analysis has demonstrated that polymorphic variation within the *BRIP1* (*BRCA Breast Cancer 1*-interacting protein 1) gene is also associated with the risk of meningioma. This association was found to be statistically significant, as suggested by case–control studies.[13] Deletion of *NFKBIA* (nuclear factor of kappa light chain gene enhancer in B cells inhibitor) has a similar effect as that in *EGFR* amplification in the pathogenesis of glioblastoma. Almost all glioblastomas have excessive activation of the epidermal growth factor receptor (*EGFR*) pathway, usually initiated by activating mutations of the *EGFR* oncogenes. A data supported a function for *NFKBIA* in the suppression of GBM tumors. This data shows that loss of *NFKBIA* can also be associated with the tumor progression and recurrence.[59]

Alterations in the tumor suppressor *p53* gene are considered to be the most common defect observed in many cancers. Results from Indian glioma patients suggested a low incidence but a definite involvement of *p53* mutations. Mutations of *p53* were detected both in low-grade (II) and high-grade (III, IV) gliomas. Their results show that the *p53* mutation frequency is higher in low-grade gliomas than in high-grade gliomas.[60] The *p53* gene is usually mutated in astrocytic gliomas in older people and genetically characterized by a high frequency of epidermal growth factor receptor (*EGFR*) gene amplification. Of all the *p53* gene mutations indicated in low-grade glioma, up to 90% of the mutations were located at *CpG* sites. A study found that the mutational status of the *p53* gene could vary within a single astrocytic glioma from wild type to several deleterious mutations.[61] A recent study reported mutations in the active site of the isocitrate dehydrogenase (*IDH1*) gene in 12% of glioblastomas. Mutations in the *IDH1* gene used to occur in majority of the younger patients.[62–64] The phosphatidylinositol 3′-kinase pathway is considered to be activated in many cancers, including GBMs, *via* inactivation of *PTEN* tumor suppressor gene. Mutations in *PIK3CA*

(PI3 catalytic alpha subunit), which is an important oncogene, were identified in a large number of glioblastoma patients.[65]

The immune system response

Immune system is vital for body's defense against disease for organisms. When the immune system is involved in engaging with bacterial load, it reduces the body's defense against cancers. Neutrophils and macrophages assemble at the site of infection and kill invaders by releasing toxic substances such as free oxygen radicals and cytokines. The immunity is either cell mediated or humoral, and it efficiently keeps the body safe and protected from invading organisms or unwanted cells or tumors. Brain and immunity work together to maintain homeostasis. There is two-way traffic of signals passing between the two, and if immunity gets suppressed or fails to identify and eliminate and unwanted colony of cells it may lead to tumor growth.[2,66–69] Cytokines have roles in both innate and adaptive immunity. As part of the innate immune response, cytokines called interferons alert other components of the immune system to the presence of cells infected with viruses. These cells are then destroyed, which limits the spread of infection. A mutation that can turn a cell into a cancerous cell is a first step in the disease process. Factors that influence whether or not cancer develops include how specialized the initial cell is and the location of that cell in the tissue. Cancer can begin at a cellular level in at least four ways: activation of stem cells that produce cancer cells, dedifferentiation, increase in the proportion of a tissue that consists of stem or progenitor cells and faulty tissue repair.[70,71]

Introduction to interferons

The role of interferons (IFNs) in cancer immunosurveillance in developing tumors is known and evident, and modern title of this function is reported as cancer immunoediting. It is evident that IFNs indeed protect the host against the tumor environment. IFNs have a positive image as the most important antiproliferative cytokine in terms of tumor elimination. The knowledge of the diversity of interferons in cancer immunity is important in understanding the pathogenesis of long-term side effects of this important cytokine, leading to impaired immune function and immunodeficiency in cancer patients.[72] Interferons were discovered in 1957 as drugs, and these were found to have antitumor and antiviral activity effects. Interferon's ability to prevent virus and nonvirus cancers in mice was discovered in late 1960s. Trials conducted by the American Cancer Society (ACS) by using recombinant DNA technology (with *E. coli*) led to the cloning and manufacture of large quantities of interferons in 1978, and by 1986 interferon alpha was approved for hairy cell leukemia. Interferons can be used as monotherapy or in combination with other interferons, cytokines, chemotherapy, surgery, radiation or hormones.[73,74] Antitumor human interferon alpha-2b (hIFN-α2b) gene is located on chromosome 9, and it encodes 19 Kda proteins with a gene size of 495 bp devoid of any intron. Interferon alpha-2b protein belongs to a class of glycoprotein that is known as cytokines, which have multifunctions after binding to the cell surface-specific receptor and result in the activation of cell signaling pathway in response to the presence of pathogens, e.g., viruses, bacteria and parasites. Interferon alpha-2b triggers the protective defense of the immune system that can suppress tumors and viruses.[75] Human interferon (IFN) is a protein messenger called cytokine produced by immune system in response to viral infection, and it is divided into several classes on the basis of the receptors on which they bind. Type-I IFNs are known as viral interferon, because they have ability to bind with a particular cell receptor that comprised IFNAR1 and IFNAR2 chains. IFN (α), IFN (β), IFN (ω), IFN (ε), IFN (ν) and IFN (κ) are included in this type. These Interferons are a primary line of defense of the host immune system against infectious and tumor development.[76,77]

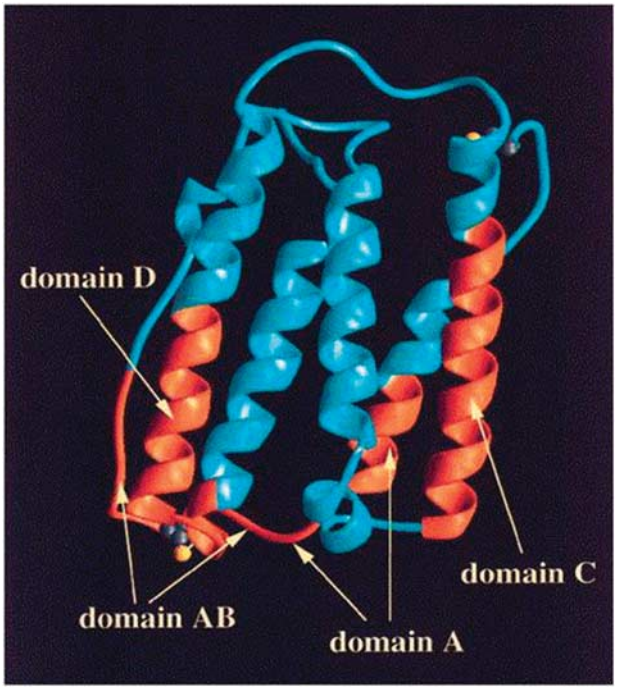

Figure 1. Structure of human interferon alpha 2b protein (Adopted from ref. 91).

IFNα has a wide range of biological activities such as antiproliferation, immunomodulation and antiviral. The human interferon alpha-2b (hIFN-α2b) protein is composed of 165 amino acids.[73,78] This protein has five α-helices (Figure 1), which are packed together in the form of helical bundles.[77,79,80] The interaction of human IFN-α2b protein with its cell surface–specific receptors initiates its action.[81] The interferon type-I has a common receptor that comprises two subunits IFNAR1 and IFNAR2 (Figure 2). Approximately 7–13% of chromosomal abnormalities are observed in the short arm of chromosome 9 in acute lymphoblastic leukemia patients where interferon alpha and interferon-beta genes are located. On chromosome 9, if the ends are deleted then, in addition to interferon genes, there are chances that there will be a loss of other tumor suppressor genes that are located at chromosome 9 ends. Various studies in different tumor types have also indicated that most of the chromosomal deletions occur on chromosome 9, which may involve interferon gene cluster.[82–84] A lack of methylthioadenosinephosphorylase (MTAP) enzyme activity along with IFN gene cluster deletion has also been observed in many cases.[85]

Interferon signaling includes heterodimerization of cell surface receptor subunits IFNAR1 and IFNAR2 with interferon alpha/beta.[86,87] As a result of binding of IFN α/β to its receptor subunits, intracellular signal transduction pathway is initiated[88,89] (Figure 2). Table 1 shows different proteins that are involved in the signal transduction pathway of human interferon alpha. The receptors for interferon α/β and interferon γ activate components of the Janus kinase–signal transducer and activator of transcription (JAK–STAT) signaling pathway, leading to the formation of at least two transcription factor complexes. STAT1 interacts with STAT2 and p48/IRF-9 to form the transcription factor IFN-stimulated gene factor 3 (ISGF3).[90] Type I IFN initiates receptor activation by bringing IFNAR-1 and IFNAR-2 together into a heteromeric complex in which the receptor chains can interact productively with each other. This process may involve a ligand-induced receptor clustering mechanism, which resembles that shown for

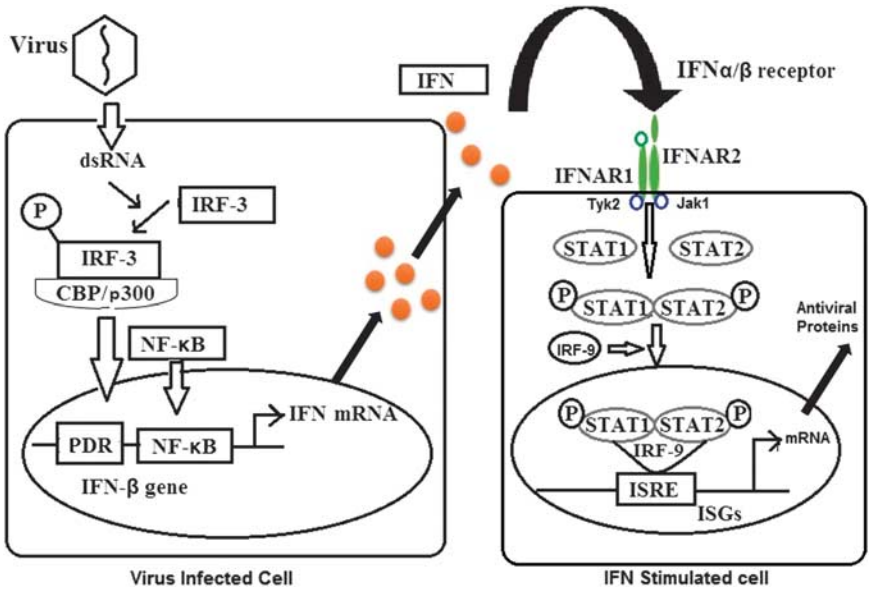

Figure 2. Signal–transduction pathways of human interferon alpha and beta (Adopted from ref. 91).

Table 1. Proteins involved in the signal transduction pathway of *human interferon alpha*[91]

Sr. No.	*Protein*	*Function*
1	Jak-1	It interacts with IFNAR2 and IFNGR1 receptors when interferon binds to the receptor.
2	Jak-2	It binds with IFNGR2 when interferon gamma binds to the receptor.
3	Tyk-2	It binds to IFNAR2 when interferon alpha attaches to the receptors.
4	STAT-1/p91	Transcriptional factor attaches to ISRE sequence and GAS sequence on DNA after phosphorylation when interferon gets attached to its receptor.
5	STAT-2/p113	Transcriptional factor and part of the ternary complex that attaches to ISRE sequence on DNA.
6	IRF-9/p48	Transcriptional factor and part of the ISGF-3 complex.
7	STAT-1α	Homodimerizes and moves to the nucleus and binds to GAS element on DNA when interferon gamma attaches to the receptor.
8	ISGF-3	STAT-1, STAT-2 and IRF-9 constitute this tertiary complex, which is attached to ISRE sequence on DNA.

Abbreviations: CBP, cyclic binding protein (cofactor for STAT protein); CREB, cyclic (adenosine monophosphate) response element-binding protein; dsRNA, double-stranded RNA; GAS, interferon-gamma-activated sequence; IFNAR, interferon alpha receptor; IFNGR1, interferon gamma receptor 1; IFNGR2, interferon gamma receptor 2; IRF, interferon response factor; ISGF, interferon-stimulated growth factor; ISRE, interferon-stimulated response; JAK, janus kinase; NF-κB, nuclear factor enhancer of activated B cells (this protein activates transcription of DNA, as well as activates B cells to produce antibodies); STAT, signal transducer and activator of transcription; Tyk, tyrosine kinase. ISGF-3: a heteromeric factor consist of four proteins p48, p84, p91 and p113. p300: cofactor for STAT protein (histone acetyltransferase). p48: a subunit of transcriptional factor ISGF3. p91: a component of the interferon (IFN)-stimulated gene factor 3 complexes. p113: also known as STAT proteins, at specific tyrosine residues.

other cytokine receptors and may reflect an allosteric conformational change in the receptor upon ligand binding. Activation of the receptor on binding of the ligand causes the Janus protein tyrosine kinase (JAK) family members Tyk2 and JAK1, which are constitutively bound to the cytoplasmic tails of IFNAR-1 and IFNAR-2, to become activated and then phosphorylate tyrosine motifs with the cytoplasmic tails of the receptor chains, which leads to the subsequent phosphorylation of STAT proteins by the JAK. These phosphorylated STAT proteins then dimerize or form ternary complexes with DNA-binding proteins—for example, p48 or CBP/p300 to form transcription activator complexes that translocate to the nucleus and promote the expression of IFN-stimulated genes (ISG).[87]

MATERIALS AND METHODS

Blood and biopsy sample collection and patient history

The brain cancer tumor samples ($n = 50$) without paraffin or formalin treatment were obtained from collaborative hospital. The patient's history will include the following parameters: name, age, sex, marital status, history of diagnoses, information of any previous treatment, tumor type, complete blood count (CBC) parameters, family history of cancer, eating habits, brain cancer staging and other associated clinical findings. The patients were investigated by CT scans and MRI (brain). The patients were operated for resection or biopsy through IGS (Image Guided Surgery) by taking necessary consents and after resection, brain tissue samples were collected in normal saline water. Samples were sent to the histopathological department for the classification of the tumor grades. Brain tumor grades were identified according to the World Health Organization (WHO) classification system. According to WHO, grades I and II were classified as low-grade brain tumors (LGBT) and grades III and IV were classified as high-grade brain tumors (HGBT).[7] Through this classification, brain tumor patients were grouped into low-grade brain tumor (LGBT) patients (n = 17) and high-grade brain tumor (HGBT) patients (n = 33). Blood samples collected from volunteers (control group) who were healthy individuals not consuming tobacco or alcohol and who were having no significant adverse exposures from the environment.

Environmental parameters and radiation measurements

The environmental parameters for brain tumor patients were recorded on a questionnaire. The following environmental parameters were analyzed:

Table 2. Background information of brain tumor patients

Parameters	*Brain tumor patients*	
	Total (n = 50)	*Percentage Prevalence (PP) %*
Age		
18–25	10	20
26–50	30	58
51 and above	10	20
Sex		
Male	33	66
Female	17	34
Marital Status		
Married	37	74
Single	13	26
Occupation/status		
Teachers/office workers	7	14
House wives	16	32
Students	5	10
Medical	1	2
Farmers	12	24
Laborers	9	18
Locality		
Rural	5	10
Siwalik	16	32
Urban-Suburban	29	58
Drinking Water		
Filtered	9	18
Unfiltered	41	82
Family History of Cancer		
Yes	10	20
No	40	80

present and past occupations, duration of occupational exposure, surrounding living environment (e.g., air quality), source of drinking water, duration of local exposure as in case of individuals exposed to radioactivity-emitting rocks/soils, economic status of the patient and other relevant environmental parameters as required (see Table 2). Some patients belonged to the Siwalik belt in Pakistan, where radiation exposure is high owing to uranium deposits and mineralization, and some patients were also residing in places in which elevated levels of gamma radiations were found from the presence of hard rocks or granite/gneisses. Radiation doses for gamma activity from these Siwalik areas were measured using gamma (γ) and X-portable prospecting high-sensitivity scintillometer (SAPHYMO-SPP2 NF; based on Sodium Iodine Scintillation Detector), and the measurement was in the range of 200–450 cps (counts per seconds), which corresponds to 0.05–0.15 μSv/hr (equivalent to: 0.4383–1.3149 mSv/y).

Hematological analysis

The hematological analysis of the brain cancer patients and control group (n = 110) was carried out by complete blood count (CBC). CBC tests were performed on Abacus+ and Medonic machines, and the following nine CBC parameters were considered: hemoglobin (HB in (g/l), white blood cells (WBC in 10^9/l), platelet count (PLT in (10^9/l), hematocrit (HCT in %), red blood cells (RBC in 10^{12}/l), mean corpuscular hemoglobin (MCH in g/l), mean corpuscular hemoglobin concentration (MCHC in %), lymphocytes (LYM) in %) and neutrophils (NEUT in %). These CBC parameters' mean values and mean values of those CBC parameters that were either below the normal range or above the normal range were calculated using the software SPSS-20. Mean values of all the above-mentioned CBC parameters were calculated for the brain tumor patient group and the control group (Table 3).

Molecular analysis

Human genomic DNA isolation from brain biopsy samples. The human genomic DNA was purified from brain biopsy samples from brain tumor patients and from blood samples taken from the control group by using the Kit Method of TIANGEN (Tiangen Biotech Beijing) genomic DNA purification kit (Cat. No. DP304). Box 1 shows protocols for DNA purification from biopsy samples and protocols for DNA purification from blood samples from the control group. The quantity of purified DNA was determined by performing 1% agarose gel electrophoresis and spectroscopic analysis on a spectrophotometer. The DNA bands in the gel were visualized from a transilluminator, and pictures were taken using the Dolphin-DOC gel documentation system.

Amplification of *human interferon alpha2b* gene

The human genomic DNA containing human interferon alpha 2b gene, of 695 bp, was amplified by using the primer set (IFN1F, IFN2R); see Table 4. PCR kit of Enzynomics (Cat. No. P025B) was used for PCR buffer reaction, $MgCl_2$, dNTPs and Taq Polymerase. The human interferon alpha2b gene was amplified from genomic DNA isolated from brain tissue samples of all brain tumor patients, as well as from the control group from blood samples by using primers as described Table 3. Table 5 shows the concentration of the PCR reaction mixture components. The primer targeting human interferon alpha 2b gene used in this study was the same as described earlier by Mahmood *et al*. (2011).[91] The human interferon alpha 2b gene was amplified from genomic DNA isolated from all patients and healthy individuals by using forward primer 5′ acttggatcctctgcaacatc-tacaatg 3′ and reverse primer 5′ taagaagcttcgtgtcatggtcatagca 3′.[91] Primer synthesis was done by Penicon, Pakistan, as well as by Advanced Bioscience International (ABI), Pakistan. The PCR reaction mixture composition is described in Box 2, and the respective protocol is shown in Box 3. The protocols for gene amplification from PCR technique were the same for both types of samples—i.e., brain biopsy samples from patients and blood samples of control group. The amplified human interferon α2b gene was further gene cleaned before sequencing using the QIA quick Gel Extraction Kit protocol.

Sequence analysis

A total of 160 (50 brain tumor patients and 110 controls) samples were found to be positive regarding human interferon alpha 2b gene amplification, and these PCR products were sent to First BASE, Singapore for sequencing. A 'BigDye Terminator v3.1 Cycle Sequencing Kit' and a 'ABI PRISM 3730×l Genetic Analyzer' (developed by Applied Biosystem, USA) were used by First-BASE, Singapore. The sequencing of PCR products was performed using both forward and reverse primers (i.e., IFN1F and IFN2R).

Bioinformatics tools for sequencing analysis

The genetic sequencing data were analyzed by using different available bioinformatics software such as Standard Nucleotide BLAST (online: http://blast.ncbi.nlm.nih.gov/Blast.cgi); Chromas LITE 2.1.1 software, which was used for peak correction analysis of DNA sequencing files; and CLUSTALW (online: http://www.genome.jp/tools/clustalw/), which was used for multiple sequence alignment to determine any point mutations in human interferon alpha 2b gene in brain tumor patients and in the control group of healthy people to draw useful conclusions on the synthesizing protein by this gene.

RESULTS

Brain tumor patients

Histopathologically confirmed brain tumor grades (n = 50) were considered for molecular analysis in this study for DNA isolations, as well as gene amplification *via* PCR technique. Among 50 samples, most reported (see Table 6) brain tumor grades were glioblastoma multiforme (n = 18; 36%), anaplastic astrocytoma (n = 15; 30%), diffuse astrocytoma (n = 9; 18%) and meningioma (n = 8; 16%). GBMs are the most aggressive tumors because of widespread infiltrative growth pattern, and meningiomas have a relatively low risk of recurrence or aggressive growth.[7,88] Histopathological photographs of GBM and meningioma are shown in Figures 3 and 4. According to American Brain

Table 3. Mean values of complete blood count (CBC) parameters for brain tumor patients and control group

CBC Parameters	*Total brain tumor patients (n = 50)*		*Control group (CG) (n = 110)*		*Normal range*
	Low	*High*	*Low*	*High*	
Hemoglobin ($g\,l^{-1}$)	10.45 (11)	—	—	—	11.5–17.0
WBC ($10^9\,l^{-1}$)	2.12 (3)	16.89 (17)	—	12.5 (2)	4.0–11.0
Platelet count (10^9/l)	123.57 (5)	538 (6)	120 (8)	—	(150–400)
Hematocrit %	32.58 (15)	—	35.46 (6)	—	36–50
RBC ($10^{12}\,l^{-1}$)	3.29 (4)	6.15 (5)	—	5.95 (10)	3.8–5.5
Mean corpuscular hemoglobin ($g\,l^{-1}$)	25.06 (23)	33.76 (5)	27.7 (32)	35 (4)	28–32
MCH Concentration (%)	30.20 (9)	37.65 (4)	31.26 (10)	36.6 (2)	32–36
Lymphocytes %	8.52 (32)	45.43 (6)	—	43.27 (22)	20–40
Neutrophils (%)	22.2 (3)	89 (24)	34.2 (2)	—	40–80

Column labeled as '*low*' shows mean of CBC parameter below normal range, and column labeled as '*high*' shows mean of CBC parameter above normal range. The number in the parenthesis describes the number of individuals whose CBC parameters were found disturbed (low or high).

Box 1. DNA isolation from tiangen kit protocols from brain biopsy samples of patients and from blood samples of controls

Step 1 (for blood samples): Blood in Eppendorf: 300 µl+TE Buffer: 600 µl = mixed (TE buffer 200 µl for next repeat)
Centrifugation: 10 000 r.p.m. (1 min)
Discard the supernatant quickly and gently
Repeat the above steps unless the color of the fluid turns white/yellow
Blood pellet: 20 µl + Buffer GA: 180 µl (mixed well) + Proteinase K: 10 µl (mixed well again)
Incubation: 56 °C (30 min)
Step 1 (for brain tissue samples):
Brain tissue: 50 µg + Buffer GA: 200 µl (mixed well) + Proteinase K: 20–25 µl (mixed well again)
Incubation: 58 °C (overnight)
Steps 2-10 (same for both biopsy and blood samples)
Step 2: Add Buffer GB: 200 µl (mixed well)
Incubation: 70° (10 min)
Step 3: Centrifugation: 6000 r.p.m. (5 sec)
Step 4: Add Absolute Ethanol: 200 µl (mixed 5-6 times)
A white precipitate may form.
Step 5: Pour the above mixture into a collection tube adjusted spin column CB3
Centrifugation: 12 000 r.p.m. (30 sec)
Discard the run-through
Step 6: Add Buffer GD: 500 µl
Centrifugation: 12 000 r.p.m. (30 sec)
Discard the run-through
Step 7: Add Buffer PW: 700 µl
Centrifugation: 12 000 r.p.m. (30 sec)
Discard the run-through
Step 8: Centrifugation of the empty column at 12 000 r.p.m. (2 min)
Discard the run-through
Step 09: Place the column into a new autoclaved eppendorf tube (1.5 ml capacity) + Injection Water: 100 µl
Incubation: Room temperature: 2–3 min
Step 10: Centrifugation: 12 000 r.p.m. (3–4 min)
Important: ***Save*** the **RUN-THROUGH** (–20 °C/ – 70 °C)
The RUN-THROUGH contains the purified DNA.

Tumor Association (ABTA),[90] meningiomas represent 34% of all primary brain tumors. Gliomas represent 30% of all brain tumors and 80% of all malignant tumors. Glioblastomas represent 17% of all primary brain tumors and 54% of all gliomas. Astrocytomas represent 7% of all primary brain tumors. Astrocytomas and glioblastoma combined represent 76% of all gliomas.[92]

Table 2 shows background information for brain tumor patients. Most of the patients were male (66%), and were mostly (58%) from the age group of 26–50 years. Most of the male patients were farmers (24%), followed by laborers (18%). With regard to location, most of the patients (58%) were from urban–suburban areas and second most of the patients (32%) were from natural-terrestrial radioactive-exposed areas. Further, 82% of the patients were consuming unsafe and unfiltered drinking water owing to either nitrate contamination because of the use of fertilizers from agricultural activities or chlorinated contamination present in tap water coming from city water supply and so on. Moreover, 80% did not report any family history of cancer.

Table 3 shows two types of columns: mean values of those CBC parameters that were below the normal range (indicated by column '*low*') and mean values of those CBC parameters that were above the normal range (indicated by column '*high*') in both, the brain tumor patient group and the control group (CG). Most significant changes were found in the following CBC parameters: LYM (lymphocytes), NEUT (neutrophils) and MCH (mean corpuscular hemoglobin), as compared with the normal range. It can be observed that CBC parameters have been more altered in brain tumor patients as compared with the controls. The most altered CBC parameter was LYM, which was found to be low in 32 patients (64%), the second most altered parameter was NEUT, which was found to be high in 24 patients (48%), and third most altered parameter was MCH, which was found to be low in 23 patients (46%).

DNA Isolations and quantifications

The human genomic DNA was purified from biopsy (patients) and blood samples (control group) by using the Tiangen genomic DNA purification kit (Cat. No. DP304). Subsequent isolations of DNA from blood samples and intact DNA band of ~15 kb were observed in all samples (Figure 5).

Human interferon alpha2b gene amplifications

The gene accession number of normal sequence of human interferon α2b was [NM-00605] and the physical location of the primer was mapped on the gene, as described in Figure 6. By using primer set IFN1F and IFN2R, the gene with signal peptide (SP) was amplified, which yielded an ~700-bp fragment that amplified the encoding region of the gene having a size of 625 bp (Figure 6).

Multiple DNA Alignments

The samples after PCR amplifications by using external and internal primer sets were subjected to sequencing after gene clean, and the peaks were corrected from sequencing file; the corrected sequences were further aligned to generate multiple alignment file by using the CLUSTALW software. The representative sequencing file showing peaks is shown in Supplementary

Table 4. Primers used to amplify *human interferon alpha 2b* gene

Sr. No	*Primer name*	*Length of bases*	T_m (°C)	*GC (%)*	*Sequence*
1	IFN1F	28	55	43	5′-acttggatcctctgcaacatctacaatg-3′
2	IFN2R	28	55	43	5′-taagaagcttcgtgtcatggtcatagca-3′

Table 5. Concentration of PCR reaction mixture components

PCR chemicals	*Stock concentration*	*Working concentration*	*Volume used (µl)*
PCR reaction buffer	10×	1×	5
$MgCl_2$	25 Mm	04 mM	2
dNTPs	2.5 mM	0.2 mM	4
Forward primer (IFN1F)	100 pmol μl^{-1}	5 pmol μl^{-1}	5
Reverse primer (IFN2R)	100 pmol μl^{-1}	5 pmol μl^{-1}	5
Genomic DNA	0.5 µg μl^{-1}	1.5 µg	5 (DNA isolated from blood sample), 7 (DNA isolated from tissue sample)
Taq polymerase	5 U	2.5 U	0.5
Distilled water			26.5 (for blood DNA) 28.5 (for tissue DNA)
Total volume			50

Box 2. Steps of mixing PCR chemicals

Step 1: Add distilled water in separate small Eppendorf tubes that are properly marked with the PCR sample number
Step 2: Add PCR buffer (in thaw form) into the same Eppendorf tubes
Step 3: Add $MgCl_2$ into the same Eppendorf tubes
Step 4: Add dNTPs into the same Eppendorf tubes
Step 5: Add F′ into the same Eppendorf tubes
Step 6: Add R′ into the same Eppendorf tubes.
Step 7: Add DNA (isolated) into the same Eppendorf tubes
Step 8: Add Taq polymerase
Step 9: Place the sample(s) into the PCR machine with the above settings

Box 3. PCR machine settings for PCR cycling conditions

Step 1: Temperature = 94 °C (3 min) [Initial denaturation]
Step 2: Temperature = 93 °C (30 sec) [Denaturation]
Step 3: Temperature = 55 °C (1 min) [Melting temperature]
Step 4: Temperature = 72 °C (1 min) [Extension]
Step 5: Temperature = 72 °C (7 min) [Final extension]
Step 6: Temperature = 4 °C - ∞ (infinite) [Reaction halted]
Step 7: Number of cycles: 35 from 93 °C (30 sec), 72 °C (1 min) for step (i) to (iv).

Figure A (Supplementary Material) for normal sequence from 1 to 498 bp. Position '1' is marked from where the gene-encoding (functional) region starts, and position '498' is mentioned where the gene's encoding region ends. The 110 samples of human interferon alpha 2b gene were amplified from healthy individuals (control group), and one normal 'N' (reference/wild-type) sequence is highlighted in green in DNA alignment (CLUSTALW). See Supplementary Box 4 in which DNA nucleotide base alignments are shown (output of CLUSTALW alignment software, 'F' for forward strand sequence and 'R' for reverse strand sequence). Supplementary Figures B-D (Supplementary Material) are screen images for peaks from Chromas Lite software showing the absence of peak in case of DNA base deletion, an insertion of a peak in case of DNA base addition and replacement of peak in case of DNA base substitution. Supplementary Box 5 (Supplementary Material) shows reference/wild-type (N) sequence of hIFN-α2b along with protein sequence.

DNA Mutation Analysis

It is known that changes in DNA are caused by mutations. All genetic variations emerge from changes in the DNA nucleotide sequences; i.e., a nucleotide change results in an amino acid change in protein sequence and thereby a change will be observed in the properties of the protein. Supplementary Table A (Supplementary Material) shows all DNA base changes that were identified in brain tumor patients along with position numbers. Table 7 analyzes most of the frequent DNA base mutations. Frequencies are calculated for those changes that had taken place more than once. No DNA mutations were observed from the control group of all 110 healthy individuals. It is observed from Table 7 that brain tumor patients show the highest percentage (8%) in DNA base substitutions; i.e., C → A at position 184, T → C at position 458 and A → C at position 498. The second highest change is observed (6%) in DNA base 'G' addition between positions 493 and 494 and base 'A' addition between positions 477 and 478. Various other DNA base changes were observed to be 4%. All mutations identified were single stranded gene mutations. Single-stranded gene mutations in hIFN-α2b have also been identified in leukemia, as reported by Mahmood *et al*,. 2011.[91]

Protein translation and alignments

Sequence of bases in DNA determines the sequence of amino acids in polypeptide, which in turn determines the shape and function of protein, and this determines the characteristics of the cell.[92] DNA strand is transcribed into mRNA, and this is further translated into protein (sequence). A change in the DNA nucleotide sequence changes the amino acid(s), and then a protein sequence is disturbed along with protein properties. DNA point mutations can proceed for catastrophic alterations in protein product of any particular gene. There may be partial loss of function of the encoding protein, total loss of the function of the encoding protein or toxic protein production to the cell.[70] Protein alignments were performed also in the CLUSTALW software. Supplementary Box 6 shows protein alignment (CLUSTALW output) with amino acids changes (CLUSTALW output) for those samples in which DNA mutations were discovered. Such protein sequence alterations are discussed in Table 8, in which characterizations were done to identify different kinds of mutations–i.e., frameshift mutations, missense, nonsense or silent mutations–caused by DNA base alterations. A total of 19 (38%) samples from

Table 6. Brain tumors grading and percentage prevalence (reported in this study)

S. No.	*Type of the tumor*	*WHO grade*	*Number of patients*	*Percentage prevalence %*
1	Glioblastoma multiforme	IV	18	36
2	Anaplastic astrocytoma	III	15	30
3	Diffuse astrocytoma	II	9	18
4	Meningioma	I	8	16

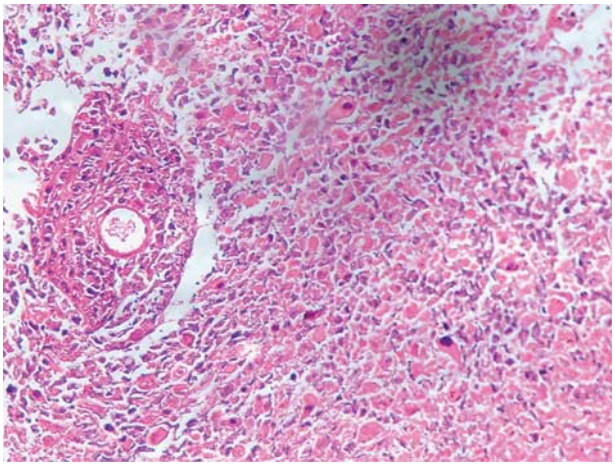

Figure 3. Glioblastoma multiforme showing endothelial cell proliferation, necrosis and mitotic activity (Photo magnification: ×40).

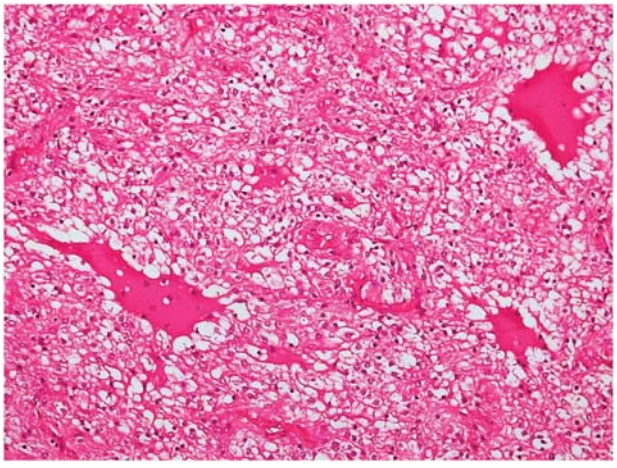

Figure 4. Meningiomas have lobulated architecture and mostly contain meningothelial whorls. They contain small to medium size syncytial cells with moderate well defined cytoplasm and short processes. Some nuclei may have grooves, while intranuclear pseudo inclusions are common. Psammoma bodies may be present. Xanthomatous degeneration, metaplasia and moderate nuclear pleomorphism may be seen but have no prognostic significance (Photo Magnification: ×20).

the brain tumor patients were reported to have mutations in DNA nucleotide sequences, and these mutations resulted in altered protein sequences with respect to altered amino acid(s).

Consequences of mutations

Table 8 describes characterization of mutations present in the protein frame; they were categorized according to the respective

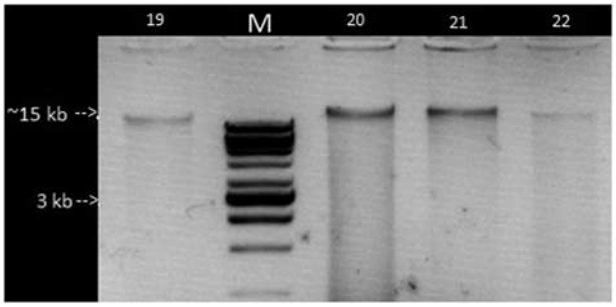

Figure 5. Representative photograph showing human genomic DNA isolation from brain biopsy samples. M: Gene ruler DNA ladder mix (Fermentas kit SM00331).

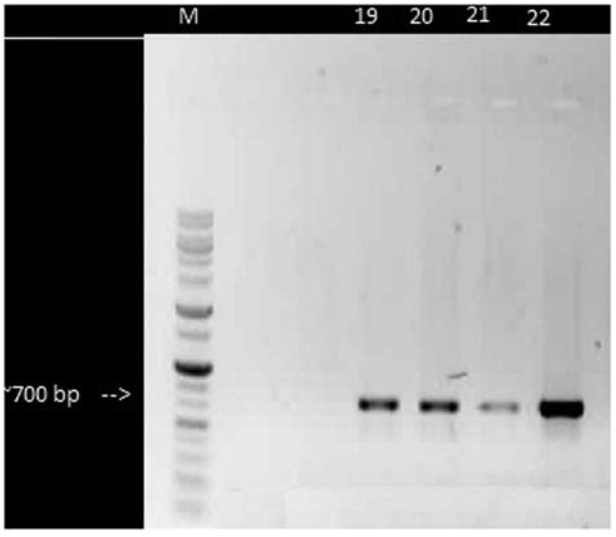

Figure 6. Representative photograph showing PCR amplification of *human interferon alpha 2b* gene by using primer set IFN1F and IFN2R, as reported earlier by Mahmood *et al.* (2011)[91] M: Gene ruler Enzynomics 1 kb DNA ladder mix (DM003).

DNA nucleotide base position change. Sample numbers with nucleotide base change i.e., addition/deletion or substitution with respect to position is mentioned which resulted into either frameshift mutations, or missense/nonsense /silent mutations respectively. Table 8 shows that brain tumor patients reported a total of 19 (38%) mutations, in which a total of 18 (36%) frameshift mutations were observed mainly because of DNA base 'C' addition between positions 53 and 54 (4%) and between 247 and 248 (4%), base 'G' addition between positions 165 and 166 (4%) and 493 and 494 and base 'A' addition between positions 195 and 196 (4%), whereas the remaining frameshift mutations were reported in 2%. There were no nonsense or missense mutations discovered, but there was only one silent mutation. Supplementary Table B

Table 7. Frequency of changes in DNA bases (most frequent mutant position)[a]

Sample No.	*Nature of genetic changes*	*Frequency*
F1, F4	C+ (52–53)	4%
F1, F4	G+ (152–153)	4%
F1, F4	A+ (182–183)	4%
R5, R54	G+ (165–166)	4%
R5, R54	C+ (295–296)	4%
R5, R54	C+ (315–316)	4%
R5, R54	T+ (406–407)	4%
R7, F25, F56	G+ (493–494)	6%
R8, R34	C+ (247–248)	4%
R8, R34	AT+ (440–441)	4%
R24, R29, R30	A+ (477–478)	6%
R24, R29	CA+ (461–462)	4%
R24, R29	G+ (487–488)	4%
R25, R56	C+ (391–398)	4%
F26, F57	A+ (195–196)	4%
F1, F4, F26, F57	C → A* (184)	8%
R7, R24	C → G* (410)	4%
R29, R30	C → A* (410)	4%
R7, R24, R29, R30	T → C* (458)	8%
R8, R34	G → C* (436)	4%
R8, R34	A → G* (437)	4%
R8, R34, R27, R28	A → C* (498)	8%
R24, R29	C → T* (417)	4%
R24, R29	T → C* (418)	4%
R24, R29	G → T* (420)	4%
R24, R29	A → T* (422)	4%
R24, R29	G → C* (423)	4%
R24, R29	G → A* (424)	4%
R24, R29	T → G* (426)	4%
R24, R29	G → T* (431)	4%
R24, R29	C → A* (441)	4%
R24, R29	T → G* (443)	4%
R24, R29	A → G* (445)	4%
R24, R29	T → A* (453)	4%
R24, R29	T → A* (454)	4%
R24, R29	C → G* (461)	4%
R24, R29	T → A* (469)	4%
R24, R29	T → A* (470)	4%
R24, R29	A → G* (473)	4%
R24, R29	A → G* (476)	4%
R24, R29	T → A* (482)	4%
R24, R29	G → A* (492)	4%
F25, F56	A → C* (15)	4%
R29, R30	T → A* (412)	4%

[a]Mutation position frequency that occurred more than once. Note: base insertion: +, base deletion: —, base replacement: *.

(Supplementary Material) shows insertion, deletion and substitution of amino acids within protein sequences. The last column of this table describes the decision of the type of mutations (i.e., frameshift, nonsense, missense or silent) on the basis of DNA base deletions/additions/substitutions. It is noticed that the following amino acids were found to be altered twice in protein sequences: L (Leucine) at positions 66 and 56, A (Alanine) at position 19 and K (Lysine) at positions 83 and 133.

DISCUSSIONS

In this study, it is identified that most of the patients were farmers (24%) and laborers (18%). Most of the patients were from urban–suburban areas (59%) of the country. Two suspected environmental factors were assessed for the brain tumor induction based on survey i.e., due to the contamination of nitrate/nitrite by agriculture practices i.e., heavy use of fertilizers etc. and chlorinated by-products in drinking water for those who were not belonged to farming but using tap water, as 80% of the patients reported unfiltered drinking water. Moreover, 32% of the patients were from areas where terrestrial radiation activity is higher owing to the presence of uranium, and therefore geological ionizing radiation (IR) is another suspected factor of brain tumor occurrence in this study. It should be noted that the groundwater consumed in these areas contains uranium (up to 100 ppb) rather than nitrates or nitrites. The fact is that (i) these areas have no chlorinated drinking water, (ii) these areas have very little cultivation and irrigation and therefore limited use of fertilizers and (iii) the groundwater is not nitrate/nitrate contaminated in these areas. It is reported in many studies that brain tumors may be caused by potential environmental factors. Agriculture practice and farming were considered to be associated with the risk of brain tumor occurrence because such activities involve the use of pesticides as well.[93] Brain cancers have been linked in other studies with rural residence and with agricultural occupations.[94,95] Pakistan is an agriculture country, and therefore both ground waters and surface waters can be considered to be contaminated by nitrate level.[96] It is known that the presence of nitrates/nitrites and/or chlorinated by-products of sewage treatment can influence the formation of a brain tumor.[7] Many epidemiologic studies have linked N-nitroso compounds (NOC) to be the factor of brain tumors.[93] It is reported in this study that a majority of the patients were not having safe drinking water, and it is evident that environmental contaminants have some impact on developing brain tumors. There may be other neurocarcinogens, and further research is required to confirm these facts. In Pakistan's cities, drinking water comes from piped water, and in villages there is a use of hand pumps, wells, open tanks and river water. Nitrate may be present in the soil and in the natural waters owing to fertilizer usage or owing to pollution from organic compounds. In polluted waters, however, levels have been reported that may have exceeded the WHO guideline for drinking water of 50 mg/l in some of the agricultural regions in Pakistan.[96,97] In Pakistan, fertilizer consumption has increased threefold during the past 30 years[98–100]; therefore, owing to heavy use of fertilizers, varying levels of nitrates are found in the underground waters.[98,101] It is also reported that nitrate contamination of groundwater has become significant in Punjab province. Recently, water quality monitoring activities in Pakistan have reported nitrate contamination in the drinking water sources and recognized it as one of the major quality issues of Pakistan. The highest percentage of contamination (23%) is found in water samples collected from both the Baluchistan and Punjab provinces.[99,101] Epidemiological research suggested the association of nitrates in drinking water and an increased risk of brain cancer occurrence, because NOC can develop cancer and there is a widespread human exposure observed from nitrates in drinking water and foods. An ecologic study conducted in Yorkshire, England, found a positive association between nitrate levels in drinking water and brain cancer development.[102] Moreover, a study based on tap water suggested the risk of astrocytoma, which may be associated with increased levels of nitrite exposures.[103] During water treatment process, chlorine-based disinfectants may interact with naturally occurring materials present in the water, and hence hazardous by-products such as trihalomethanes (THMs) and halogenic acetic acids (HAAs) may be formed.[104] A research supported by National Cancer Institute (NCI), Bethesda found a dose–response relationship among humans between brain cancer risk and the duration of consuming high levels of chlorinated tap water. There was little association of glioma with the tap water intake as compared with farming and rural residence.[105]

Many researchers have investigated potential environmental risk factors for brain tumor incidence, and the most consistent factor was the ionizing radiation (IR) exposure.[12] It is reported here that 32% of patients dwelled in radioactive-exposed areas (Table 2). Natural radiations are established risk factors that are

Table 8. Frequency of gene *human interferon alpha 2b* mutations

	Sample No.	*DNA base position no.*	*Nature of mutational change*	*No. of mutations*	*Frequency %*	*Most frequent amino acid change position* [a]
	F1, F4	(C+, 53–54)	Frameshift mutation	18 (36%)	4%	A(19) = 2
	R5, R54	(G+, 165–166)			4%	L(56) = 2
	R7	(G+, 406–407)			2%	K(83) = 2
	R8, R34	(C+, 247–248)			4%	K(133) = 2
	R24	(TG−, 414–415)			2%	L(66) = 2
Brain tumor patients ($n = 50$) Total number of mutations: 19 (38%)	F25, F56	(G+, 493–494)			4%	
	R25	(C+, 397–98)			2%	
	F26, F57	(A+, 195–196)			4%	
	R27	(T+, 487–488)			2%	
	F28	(C+, 31–32)			2%	
	R29	(CA+, 461–462)			2%	
	R30	(TTAC+, 406–407)			2%	
	R56	(C+, 397–398)			2%	
			Nonsense mutation	0	0%	
			Missense mutation	0	0%	
	R28	(A → C,* 498)	Silent Mutation	1	2%	

Note: base insertion: +, base deletion: —, base replacement: *. [a]Occurred more than once.

reported to induce brain cancer.[106] The Siwalik belt of the lower hilly areas contains very large number of radioactive anomalies and a number of uranium mineral deposits.[47,50] A study[107] has revealed that the mean value of the environmental gamma radiation dose rate level in Pakistan is of the order of 59nGy/h, which is higher than the worldwide mean value of 44nGy/h. It is well known that ionizing radiation can cause DNA damage through the generation of free radicals or through ionization of the DNA; hence, proliferative cells can undergo apoptosis.[108] Humans are naturally exposed to terrestrial radionuclides (for example, uranium, thorium, their decay products and potassium-40), which may be present in the air, water and agricultural areas where they contaminate the growing food. Moreover, radioactive contaminated building material can accumulate radon gas and its decay products inside houses, and inhalation of such radionuclides can pose health hazards.[109] It is found that 200-450 cps (counts per seconds) of scintillometer which corresponds to 0.015-0.15 μSv/hr was found in the Siwalik belt areas.[49,50] Radioactivity-exposed residents are considered to be under the stress of uranium effects. Adverse health effects have been noted from uranium exposure, because uranium has radiological toxicity. Most significantly, high exposed levels of uranium can induce cancer owing to high affinity of uranium with the DNA. Genotoxic effect initiates through the absorption of high levels of natural radiations.[110] Uranium particles that bind to the DNA amplify the impacts from such radiations by photoelectron enhancement effect (PHE).[111]

Ionizing radiations have significant effects on human tissues; they affect the DNA bases that constitute genes, and thus they cause brain tumor.[112] The increased incidences of malignant brain tumors reported in several studies are attributed to environmental factors and occupational exposure.[20] Ionizing radiation is an established risk factor for brain cancer, as reported by Sadetzki *et al.* (2005).[106] X-radiation and gamma radiation have been shown to cause a broad spectrum of genetic damage, including gene mutations, minisatellite mutations, micronucleus formation, chromosomal aberrations, ploidy changes, DNA strand breaks and chromosomal instability. Genetic damage by X-radiation or gamma radiation has been observed in humans exposed accidentally, occupationally or environmentally. Epigenetic mechanisms that alter the action of genes may also be involved in radiation-induced carcinogenesis.[113] Health effects of exposure to uranium can result from chemical and radiological toxicity of uranium. The most remarkable damage caused by uranium is cancer. Uranium has a high affinity to DNA, which results in abnormally high absorption of natural background radiation and accelerates genotoxic effects.[110] This advanced biochemical and biophysical aspect of uranium contamination is described as photoelectron enhancement effect. Therefore, uranium particles bound to DNA amplify effects from external irradiation.[111]

This study assessed the role of an immunomodulatory, antiviral and antiproliferating gene (i.e., human interferon alpha-2b) with respect to the number of mutations discovered in brain tumor patients. DNA base substitutions can result in missense, nonsense or silent mutations, whereas DNA base insertion or deletion can induce frameshift mutations. A missense mutation changes protein slightly, it is a change in DNA sequence that changes the codon with different amino acid, it forms unpredictable protein function. A nonsense mutation blocks protein production with a stop codon rather than an amino acid, resulting in a premature shortening and truncation of protein (incomplete protein). A silent mutation does not affect the protein (normal protein) because it does not alter the specified amino acid. Frameshift mutations change all amino acids and develop a nonfunctional protein product that is different from the normal protein production (abnormal nonfunctional protein). Moreover, reading frames in frameshift mutations contain stop codons that result in the truncation of the mutation protein prematurely. The reading frame along the mRNA is shifted, resulting in altered amino acids and premature termination.[70,114,115] The immune system protects the human body by eliminating pathogens and abnormal cells with minimal damage to healthy tissues.[116] Immune impairment is linked to cancer development primarily, which further expands with progression to metastatic disease. A study hypothesized that impaired IFN signaling may be a common immune defect in cancer patients.[117] Inside the human body, the human interferon alpha 2b protein attaches to its receptor IFNAR1 and IFNAR2 and initiates an anticancerous and antiviral signal transduction pathway of Jak and STAT proteins, resulting in the activation of more than 30 different types of antiviral and anticancerous genes.[91] Interferon is a cytokine secreted by almost all eukaryotic cells that are exposed to virus, bacteria, mitogen and antigen. The secreted interferon stimulates surrounding cells to

produce other proteins, which regulate viral replication, immune response, cell growth and other cell functions.[73] Interferon alpha shows a front-line defense mechanism for the host against viral infection, microorganisms foreign cells, foreign macromolecules or various other chemical compounds.[118,119] Interferon alpha is a member of the interferon family, comprising a large group of multifunctional secreted proteins that have antiviral, immunomodulatory and antiproliferative activities; these proteins also regulate basic cellular functions including growth, differentiation and exhibit antiproliferative activity on normal, as well as on transformed, cells and control signal transduction and immune response modulation.[120–122]

It is evident from the present molecular analysis that brain tumor patients were found to have various mutations in this gene (hIFN-α2b) and especially increased number of frameshift mutations (i.e., 36%). Moreover, most of these patients' (64%) hematological profiles through CBC reports have shown a decline in 'lymphocytes' parameter, which verifies and affirms that brain tumor patients have an impaired immune system response. Moreover, a rise in neutrophils was found in 48% of the patients. These facts can be combined to state an immunomodulation initiation in brain tumor patients. That is to say that these patients were compromising their immune system as evident from mutations in this antiviral and antitumor gene. This study affirms the clinical suspicion that brain tumor cells may be modulating host immune response. Brain tumor and various other factors such as chemotherapeutic drugs, other toxins and ionizing radiations can cause an inflammatory response and hence the rise in inflammatory cells. Lymphopenia in nervous system tumors was first reported by Bill and Morgan in 1970s in children who were diagnosed with neuroblastomas.[123] Lymphopenia has been reported in meningiomas as well. Low levels of MCH are linked and signify anemia in cancer patients owing to decreased production of functional red blood cells.[124] Brain cancers suppress the hematopoietic system through bone marrow infiltration or production of factors—i.e., cytokines that may reduce the production of red blood cells as well.[125,126] It was reported in a study that anemia is frequently ($>40\%$) observed in cancer patients.[127] Lymphopenia is observed in glioma patients is a factor of the down-regulated systemic immune response.[125]

The type I interferons are central coordinators of tumor–immune system interactions and have a function in cancer immunosurveillance and immunoediting. A study by Dunn *et al.* (2006)[128] shows that type I IFNs participate in naturally occurring, protective immune responses to primary tumors.[128] Troemel *et al.* (2006)[129] observed that *p38 MAPK* (mitogen-activated protein kinases) contributes to the enhanced longevity of daf-2 mutants, showing *p38 MAPK* signaling in the regulation of longevity, owing to its role in immunity.[129] Scientists have found that cancer-initiating cells that result in glioblastoma multiforme suppress an immune system response. Glioblastoma and other cancer patients have impaired immune responses. The cancer stem cells inhibit T-cell response, and these T cells detect and destroy cancer.[130] Glioblastoma stem cells suppress T-cell responses in different ways—i.e., by reducing immunosuppressive cytokines that combat the responses of T cells, by inducing T cells to turn into regulatory T cells and by killing T cells through apoptosis. This is accomplished through the immunosuppressive protein *B7-H1* in the stem cells directly contacting the T cells.[130]

Infection in the body elicits inflammation, and a cascade of changes in cytokines such as interleukin-1 (*IL-1*) and tumor necrosis factor-a (*TNFa*) come into play. The inflammatory area informs the brain *via* neurological tracks. The basal ganglia and circumventricular structures of the brain respond through a slow transmission pathway by the release of cytokines through choroid plexus.[67,131] Glioblastoma multiforme patients have been profoundly observed to have immunosuppression. A group of researchers proposed that those strategies that induce an altered differentiation condition of cancer-initiating cells may be used to reverse immunosuppression and therefore regarded as an immunotherapy approach.[130] Some researchers hypothesized that transfection of glioma cells with type I IFN genes and provision of dendritic cells would promote effective antitumor activity by facilitating apoptosis of glioma cells and the presentation of the glioma antigens. Therefore, induction of specific immune responses against glioma cells is achieved.[132–134] A study demonstrated immune patterns in previous GBM genomic analyses and suggested the involvement of immune cells in GBM. They found that patients with proneural GBM had longer survival time, and therefore they concluded that the antitumor immune response could have more time to occur and slow down the tumor progression with a particular immune response profile.[135] Recently, it is investigated that in order to promote an effective immunotherapy against glioma, viruses have also been engineered for targeted delivery and expression of cytokines that trigger, activate and recruit immune effectors to the tumor, and as a result it showed a significant antitumor effect.[10,134] An important study by Critchley-Thorne *et al.* (2009)[117] had determined that altered IFN signaling is a general mechanism of immune function in patients with cancer of different grades. They hypothesized that an altered IFN signaling may be a significant mechanism of immune dysfunction, which is common to any cancer. In their study,[117] they identified an immune defect in three major types of cancer (i.e., breast, melanoma and gastrointestinal). They demonstrated that IFN-α signaling is declined in T and B cells, and IFN-γ signaling is declined in B cells, from patients of breast cancer, melanoma and gastrointestinal cancer. They also demonstrated that in cancer patients an impaired IFN signaling is an early and consistent mechanism of immune dysfunction.[117]

It is suggestive here that various other key genes in brain tumor must combine to infer the potential after-effects from this disease for further mitigation for this disease. Of course, a chemotherapy or radiotherapy is suggested after diagnosis related to brain benign grade or malignant high-grade tumor. Moreover, selection of an immunotherapy of glioma patients is a serious challenge because grade IV GBMs have a poor and resistive response to chemotherapy and radiotherapy. It is recommended that immunological examination of the suspected individuals who are at risk of brain cancer should be conducted because in this disease an immune suppression is observed. Glioblastoma multiforme, a lethal cancer that has no cure and responds poorly to radiotherapy and chemotherapy, is associated with immune suppression and evasion, and patients die within an year of diagnosis.[130,134,136–138] Therefore, there is a need to develop novel therapeutic drugs to treat GBMs. Immunotherapy is used to support the immune system to detect and destroy tumor cells. Such therapies activate the immune system that recognizes tumor cells even from distant sites from the primary tumors.[67] Central nervous system is regarded as an immune-shielded area from systemic immune responses and hence promotes immune evasion of tumorous cells.[10]

The concept of using the immune system as a therapeutic option has been suggested for many decades by controlling adaptive immune mechanisms. Immunotherapy could provide a durable and targeted treatment against GBMs. For new immunotherapeutical techniques to be successful, there is a need to overcome immunosuppression through immune checkpoint signaling.[138] Immunotherapy stimulates and trains the patient's immune system to detect and destroy malignant tumor cells. A study indicated that drugs that act as an immune response modifier, which has antiviral and antitumor activities, are found to be useful for the destruction of CNS-1 glioma tumor cells.[116] It is generally believed that brain tumors have low immunogenicity and suppress the immune response, and CNS has disadvantage to augment immune response. However, recent understanding of cytokine action and in gene delivery techniques promoted an

interest in immune gene therapy by using a variety of cytokines that can increase tumor immunogenicity or trigger the host immune response.[139] An article outlined the clinical application of interferon-β for treating brain tumor. The glioma-derived endogenous IFN-β has an antitumor effect on human glioma cells. Yoshida *et al.* (2004) has been developing IFN gene therapy that locally generates a large amount of endogenous IFN-β by introducing liposome into glioma cells.[140] Research by Wei *et al.* (2010) had concluded that cancer-initiating stem cells contribute to tumor destruction of the immunosurveillance, and approaches that alter the differentiation state may reserve an immunotherapeutic.[130]

CONCLUSION AND FUTURE RECOMMENDATIONS

Environmental factors and neurocarcinogens have been reported as risk factors of brain cancer occurrence because human genes are affected from different environmental conditions. This study pointed out intake of unfiltered water (possibly due to the presence of nitrated or chlorinated by-products) in most of the brain tumor patients. Moreover, 32% of patients were inhabitants of regions where terrestrial ionizing radiations were present as a result of radioactivity-emitting rocks and soils from uranium deposits and its mineralization. The above-mentioned findings related to suspected environmental causes were survey-based; further research is required to affirm these factors. It is evident from the molecular analysis that both low-grade and high-grade brain tumor patients were found to have various mutations in an immunomodulating *human interferon alpha-2b* gene, especially increased number of frameshift mutations (36%) were observed. Glioblastoma multiforme (GBM) patients are notable for profound immunosuppression, and it is known that GBM-linked cancer-initiating cells contribute to the immunosuppression. Moreover, brain tumor patients' hematological profiles have also indicated that most of the them (64%) were having depressed 'lymphocytes', which verifies and affirms that brain tumor patients have an impaired immune system response. These patients were compromising their immune system as evident from mutations in this antiviral and frontline defense control gene. It is generally known that brain tumors suppressed the immune response. The role of type I interferons is evident as immunosurveillance and immunoediting in cancers, as they have an important role for interactions between tumor and the immune system. It is suggestive here that various other key genes (tumor suppressor genes, oncogenes and DNA repair genes) in brain tumor must also be correlated to infer the possible after-effects from this disease for further investigations of new drugs. Chemotherapy or radiotherapy is suggested after diagnosis of benign brain tumor grade or malignant high-grade tumor. However, a selection of an immunotherapy of glioma patients is a serious challenge because grade IV GBMs have a poor and resistive response to chemotherapy and radiotherapy because still there is a limited success related to the use of immunotherapeutic drugs. Resistance of GBM to chemotherapy and/or radiotherapy is a serious challenge and also limits the success related to the use of immunotherapy drugs. This study indicates that mutational analysis (in an immune response gene) may be helpful to develop certain biomarkers that may be used to develop novel immunotherapeutical drugs that enhance a better immune response to fight against cancer disease and to prolong a patient's life to some extent. There is a recommendation of regular checkups of radiation-exposed people along with monitoring of all blood parameters in order to diagnose an early change in their entire health. There are long-term impacts of low dose ionizing radiations on the immune functions in human health. Further, some suggestions are: (i) the ground water from various aquifers in such areas should be analyzed for uranium and safe aquifers should be exploited for drinking water or for agricultural use; (ii) the building designs should be prepared to reduce the Radon exposure accumulation; (iii) most of the crops in these areas are considered as rain-fed therefore, only those crops should be grown which may accumulate uranium in their roots.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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