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Original Article

Mutations of the human interferon alpha-2b (hIFNα-2b) gene in cancer patients receiving radiotherapy

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Abstract: This research aimed to find out the impact of ionizing radiations on the hIFNα-2b gene of radiotherapy treated cancer patients. The gene hIFNα-2b synthesizes a protein which is an important anticancerous and antiviral protein. The cancer patients (breast, lung, thyroid, oral and prostate) who were undergoing a radiotherapy treatment were selected. A molecular analysis was performed for DNA isolation and gene amplification through PCR, to identify gene mutations. Further, by bioinformatics tools we concluded that how mutations identified in gene sequences have led to the alterations in the hINFα-2b protein in radiotherapy receiving cancer patients. The 32% mutations in the hINFα-2b gene were identified and all were frameshift mutations. Radiotherapy can impact the immune system and cancer patients may modulate their immunity. Understaning the mechanisms of radiotherapy-elicited immune response may be helpful in the development of those therapeutic interventions that can enhance the efficacy of radiotherapy.

Keywords: Interferon (IFN), immunity, radiotherapy, mutations, ionizing radiations

Introduction

This study was aimed to find mutation(s) in an immune-response gene (hINFα-2b) in different cancer patients (breast, lung, thyroid, oral and prostate) during 2013-2014. It is known that ionizing radiations (IRs) such as X-rays or gamma-rays can disrupt base pair sequences in DNA molecules. IR is known to induce gene mutations (deletions, insertions and point mutations), meanwhile further activation of cellular defensive mechanisms occurs in the body. An addition or deletion in a DNA base can demolish a gene's information with a further alteration in the encoded protein's amino acid sequence [1]. The damaged signals may be transmitted from irradiated to non-irradiated cell, which leads to the occurrence of biological effects (bystander effect) in cells that are not exposed to radiation [2-6].

Radiotherapy is the use of IR in the treatment of malignant tumors [7] and approximately half of cancer patients receive it. It is estimated that 40% of the cancer patients are cured by radiotherapy as compared to the cure of 49% by surgery and 11% by chemotherapy [8]. The diagnostic imaging, such as computed tomography (CT), magnetic resonance imaging (MRI), positron emission tomography (PET) and molecular imaging techniques, has led the development of conformal radiotherapy (CRT) and intensity modulated radiotherapy (IMRT) techniques [9-11]. The use of stereotactic radiation treatment, particularly 'single high dose gamma-knife therapy' has increased [12]. Radiation therapy treatment mostly involves high-energy radiation (external beam radiation) such as X-rays, gamma rays and charged particles to reduce a tumor followed by killing the cancerous cells. Three dimensional conformal radiation therapies (3D-CRT) involve the imaging to scan pictures and mapping the tumor location in three dimensions. The intensity modulated proton therapy (IMPT), involves the usage of the proton beams, whereas, a stereotactic radio-

surgery (SRS) and fractionated stereotactic radiotherapy (FSR) can distribute a large and a precise dose of radiation to a small and a well-defined tumor [13].

Radiotherapy treatment involves the planning of total radiation dose, dose/fraction, number of fractions and volume to be irradiated [8]. The local irradiation of some of the tissues can be produced by using radioactive nuclides embedded in the tissue by the usage of needles of ^{90}Sr, ^{90}Y and pellets of ^{198}Au etc. [10, 11]. In radiotherapy procedure, the source of radiation absorbed doses outside a treated volume is followed from the leakage through the machine and scatter from the collimator and the beam modifiers [14, 15]. It is reported that radiotherapy procedures with the latent period of about 10-15 years, can lead to substantially increased risks of the cancers of breast, skin, thyroid, parotid, parathyroid and brain [12, 16]. Because in radiation therapy, out-of-field organs may receive very low doses of radiation and it is well known that even a small radiation dose to these organs can cause cancer [17]. The impact of the primary dose distribution on secondary cancer incidence was analyzed and it was found that there is a moderate risk for the secondary cancer induction using modern treatment techniques such as IMRT [18]. Secondary cancers originate when an injury to the DNA of irradiated cells occurs and genetic programming is altered to raise an abnormal cellular growth and proliferation [19].

The radiogenomic studies are conducted to determine which polymorphism impact radiosensitivity and to estimate severe complications resulting from a radiotherapy treatment [20]. Various studies have explored variation in human genes related to the biological response to ionizing radiations. Substantial effort has been made to discover genetic markers and those single nucleotide polymorphisms which are associated with radiation that can render adverse response from radiotherapy. The microsatellite markers associated with acute adverse effects of radiotherapy in cancer patients have been identified from genome-wide screen based studies [21]. The presence of mutations (58%) of the *TP53* (tumor protein53) gene was investigated in secondary cancers arose from the irradiation field of a primary tumor following radiotherapy [22]. The researchers have evaluated the role of polymorphisms in *GST* (glutathione S-transferase) genes *GSTM1, GSTT1, GSTA1* and *GSTP1* in predicting acute side effects of radiation therapy on normal tissue. This data indicated that *GSTP1* plays an important role in shielding normal cell radiation damage [23]. A study revealed those pathways which are involved in radiation response and encompass a multitude of genes of which they had selected 11 candidate genes (*CDKN1A* (*p21*), *TP53*, *ATM*, *HDM2*, *TGFB1*, *XRCC1*, *XRCC3*, *XRCC4*, *XRCC5* (*Ku80*), *PRKDC*, and *LIG*) for their presumed or demonstrated influence on radiosensitivity [20].

The type-I interferons (IFNs) are known to participate in 'immunosurveillance' and 'immunoediting' in cancers as they are the chief administrators of the tumor-immune interactions. The cytokines (e.g., interferons) alert other segments of the immune system to the cells infected with viruses to destroy and then terminate the infection spread [24-26]. Antitumor (hIFNα-2b) gene is located on chromosome 9 and encodes 19 Kda proteins with gene size of 495 bp devoid of any intron [25, 27]. Interferon alpha-2b protein is a class of glycoprotein known as 'cytokines' having multifunctions after binding to the cell specific receptor resulting in the activation of cell-signaling pathway in response to the presence of viruses, bacteria, and parasites etc. The hIFNα-2b gene prompts the defense of the immune system that suppresses tumors [25, 27, 28]. Trials were conducted by the American Cancer Society (ACS) and large quantities of interferons in 1978 and by 1986 were manufactured and then interferon alpha was approved for hairy cell leukemia [29]. Human interferon (IFN) is a protein messenger (cytokine) produced based on receptors on which they bind. Type-I IFNs are known as viral interferon, they have ability to bind with a particular cell receptor that constitutes IFNAR1 (Interferon alpha receptor 1) and IFNAR2 (Interferon alpha receptor 2) chains. The interferons are the primary line of defense of the host immune system against infections and tumor development [25, 30, 31].

Interferons are most important anti-proliferative cytokine for tumor elimination. The knowledge of the diversity of interferons in cancer immunity is important in understanding pathogenesis of long term side effects which can lead the damaged immune system or immunodeficiency in cancer patients [32]. Interferon-alpha has wide biological activities in anti-proliferation and immunomodulation. Human

Table 1. Background Information of Radiotherapy Treated Cancer Patients

Parameters		Radiotherapy Treated Cancer Patients (n=25)	
			PP* %
Age	10-25	6	24
	26-50	8	32
	51 & above	11	44
Sex	Male	15	60
	Female	10	40
Marital Status	Married	19	76
	Single	6	24
Occupation	Farmers	2	8
	Laborers	5	20
	Field Workers	5	20
	Teachers/Office Workers	5	20
	House wives	6	24
	Students	2	8
Drinking Water	Filtered	4	16
	Unfiltered	21	84
Cancer Type	Breast	8	32
	Prostate	2	8
	Oral	5	20
	Thyroid	7	28
	Lung	3	12
Locality	Urban/Sub urban	15	60
	Rural	10	40

*PP percentage prevalence.

interferon alpha-2b (hIFNα-2b) protein comprised of 165 amino acids [25, 33]. This protein (hIFNα-2b) has five α-helices and are packed together in the form of helical bundles [25, 31, 40, 41]. The interaction of human IFN-α2b protein to its cell surface specific receptors begin its action [34]. The 7-13% chromosomal abnormalities are observed in the short arm of chromosome 9 in the patients of acute lymphoblastic leukemia, where IFN-α and INF-β genes are located. The most chromosomal deletions occur on chromosome 9 which may involve interferon gene cluster [35-39].

The mechanism of immune dysfunction in cancer includes following: defects in antigen recognition (first signal), co-stimulation (second signal), and IFNs (third signal) for efficient natural killer cell-mediated cytotoxicity [41]. An important study [41] had identified an immune defect common to three major types of cancer (breast, melanoma and gastrointestinal). These researchers [41] have demonstrated that IFN-α signaling is reduced in T and B cells, and *IFN*-γ signaling is reduced in B cells in patients with breast cancer, melanoma, and gastrointestinal cancer. It was demonstrated that an impaired IFN signaling is an initial and a constant mechanism of immune dysfunction in cancer patients. Therefore, they demonstrated that IFN signaling is impaired in lymphocytes from cancer patients. Such findings will further lead to the development of novel strategies in order to improve immunotherapeutic strategies [41].

Patients and methods

Blood sample collection and background Information

Blood samples (3-ml) for molecular analysis and Complete Blood Count (CBC) reports were collected with informed consents from the collaborative hospital's cancer patients (n=25) who were undergoing radiotherapeutical treatment for the tumor of breast, lung, thyroid, oral and prostrate. These patients were not being treated with chemotherapy when blood sampling was conducted. Blood samples were also collected from volunteers (control group n=50) for molecular analysis and Complete Blood Count (CBC) reports by taking willingness to participate in this research. The control group (healthy individuals) and the patients were age-matched and had the same environmental and socioeconomic backgrounds. The cancer patients as well as the control group had balanced food intake, were non-alcoholic and non-smokers. All individuals reported no family history of cancer. Information regarding age, sex, marital status, radiotherapy staging, dietary habits, drinking water, locality and occupation were taken on a designated proforma and some of the combined results are shown in **Table 1**.

Hematological analysis

The hematological analysis of 25 cancer patients (breast, lung, oral, prostate and thyroid) and control group (n=50) were carried out by complete blood count (CBC). The following

Table 2. Mean values of complete blood count (CBC) for radiotherapy treated cancer patients and controls

S. No.	CBC Parameters	Radiotherapy Treated Cancer Patients (n=25)		Control Group (n=50)		Normal Range
		Low	*High*	*Low*	*High*	
1	Hemoglobin-HB (g/l)	9.55 (12)	-	-	-	11.5-17.0
2	White Blood Cells-WBC (10^9/l)	2.73 (6)	13.61 (2)	-	12.6 (2)	4.0-11.0
3	Platelet Count-PLT (10^9/l)	114.53 (2)	541.25 (4)	120.22 (8)	-	150-400
4	Hematocrit-HCT %	29.11 (10)	-	35.50 (6)	-	36-50
5	Red Blood Cells-RBC (10^{12}/l)	3.33 (9)	-	-	5.94 (10)	3.8-5.5
6	Mean Corpuscular Hemoglobin-MCH (pg)	25.34 (12)	32.7 (1)	25.7 (32)	35.1 (4)	28-32
7	MCH Concentration-MCHC (g/dl)	30.96 (10)	-	31.28 (10)	36.68 (2)	32-36
8	Lymphocytes-LYM %	11.47 (11)	43.6 (4)	-	43.26 (22)	20-40
9	Neutrophils-NEUT (%)	21.32 (4)	82.8 (3)	34.21 (2)	-	40-80

The column labeled as "*low*" shows mean of a CBC parameter below normal range, the column labeled as "*high*" shows mean of a CBC parameter above normal range and the column labeled as "*total*" shows mean of a CBC parameter of each individual. The number in the parenthesis describes the number of individuals whose CBC parameters were found altered (low or high).

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 pg, mean corpuscular hemoglobin concentration (MCHC) in g/dl, lymphocytes (LYM) in % and neutrophils (NEUT) in %). These CBC parameters' mean values and mean values of those CBC parameters which were either below the normal range or above the normal range were analyzed by using software SPSS-21 by the method of 'Descriptive Analysis' for cancer patients and the control group (**Table 2**).

Molecular analysis

Human genomic DNA isolation from blood samples: The human genomic DNA was extracted from the blood samples of cancer patients and from the control group by using Kit Method of TIANGEN (Tiangen Biotech Beijing) genomic DNA purification kit (DP304). The quantity of purified DNA was determined by doing 1% agarose gel electrophoresis and spectroscopic analysis on a spectrophotometer. The DNA bands in the gel were visualized from transilluminator and the photographs were taken using Dolphin-DOC gel documentation system [25].

Amplification of human interferon alpha2b gene: The human genomic DNA containing hIFNα-2b, of 695 bp, was amplified by using primer set (IFN1F, IFN2R). The PCR-kit of Enzynomics (P025B) was used for PCR buffer reaction, $MgCl_2$, dNTPs and Taq Polymerase. The hIFNα-2b gene was amplified from genomic DNA isolated from the blood samples of radiation treated cancer patients as well as from the control group by using primers. The primer targeting hIFNα-2b gene used in this study was same as described earlier by Mahmood et al. [27]. The hIFNα-2b gene was amplified from genomic DNA isolated from all patients and healthy individuals by using forward primer 5' acttggatcctctgcaacatctacaatg 3' and reverse primer 5' taagaagcttcgtgtcatggtcatagca 3' [27]. The primers' synthesis was done by Penicon, Pakistan. Three different PCR cycles were analyzed for optimization with different melting temperatures ranging 50°C-60°C. Remaining cycling condition were as follows: initial denaturation at 94°C for 3 minutes, coupled with 35 cycles of denaturation at 93°C for 30 seconds, melting temperature 55°C for 1 minute, extension at 72°C for 1 minute and final extension at 72°C for 7 minutes. The reaction was suspended by the PCR machine to 4°C. The amplified PCR products were checked on 1% agarose gel for validation (**Figure 1**). The amplified hIFNα-2b gene was further gene cleaned prior sequencing grade by QIA quick Gel Extraction Kit protocol [25].

Sequence analysis and bioinformatics tools: A total of 75 (25 cancer patients and 50 controls) samples was found positive regarding hIFNα-2b gene amplification and these PCR products were sent to First BASE, Singapore for sequencing. Sequencing of PCR products were per-

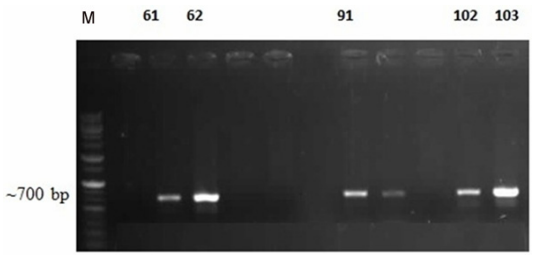

Figure 1. Representative photograph showing PCR amplification of human interferon alpha 2b (hIFNα-2b) gene by using a primer set IFN1F and IFN2R [27]. Gene ruler (M): "Enzynomics" 1 kb DNA ladder mix (DM003). Samples (61, 62, 91, 102, 103) are represented on right side.

formed using both forward and reverse primers (i.e., IFN1F, IFN2R) [25]. The genetic sequencing data was analyzed by using bioinformatics softwares such as BLAST-Basic Local Alignment Tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi), Chromas LITE 2.1.1 for peak correction analysis of DNA sequencing files and CLUSTALW (http://www.genome.jp/tools/clustalw/) for multiple sequence alignment to determine any point mutations in human interferon alpha 2b gene [25] in cancer patients as well as in the control group to draw useful conclusions on the synthesizing protein by this gene.

Results

Background information

Background information for cancer patients indicated in **Table 1**. These cancer patients were not treated prior with chemotherapy at the time of sampling. They were being treated with the radiation therapy for tumor(s) and the specific doses were given to the tumor site(s). Patients with breast (32%), oral (20%), thyroid (28%) lung (12%) and prostate (8%) cancers were selected. For breast and lung cancers the optimal dose was 60 Gy/37 Fr, for prostate cancer, it was 78 Gy/37 Fr, for oral cancer, it was 70 Gy/37 Fr and for thyroid cancer it was 30 Gy/37 Fr. The majority of the cancer patients was in the last stages of their respective radiotherapy treatment. These cancer patients were mostly male (60%) and of age group '50 and above' (44%) and majority of these were laborers (20%), field workers (20%) or office workers (20%). Field workers were mostly associated with electricians or technicians. The majority of the females was housewives. The majority of the patients (84%) were not having safe drinking water. Most of the patients reported from urban/suburban localities.

Hematological profiles

Most affected CBC parameters were: Low HB (48%) and low MCH (48%) in radiation treated cancer patients as compared to the controls (**Table 2**). The second most affected parameter was low LYM (44%), and the third most affected parameters were low HCT (40%) and low MCHC (40%). Overall, eight CBC parameters: HB, WBC, HCT, RBC, MCH, MCHC, LYM and NEUT were below the normal range and one CBC parameter PLT was in the high range in cancer patients. Majority of the patients had their CBC parameters affected because of the ailment and radiation treatment as compared to the control group. Decreased trend of LYM, HB, MCH, MCHC and HCT signify depressed immune response and anemia in cancer patients.

DNA isolations, quantifications and human interferon alpha2b gene amplifications

The human genomic DNA was purified from blood samples by using Tiangen genomic DNA purification kit (Cat. No. DP304) [25]. Subsequent isolations of DNA from blood samples and intact DNA band of ~15 kb was observed in all samples. The gene accession number of the normal sequence of hIFNα-2b is 'NM-00605' [25]. By using a primer set IFN1F and IFN2R [25], the gene with signal peptide (SP) was amplified, which yielded ~700 bp fragment which amplified encoding region of the gene having size 625 bp (**Figure 1**).

Multiple DNA alignments

The samples of both cancer patients and control group after PCR amplifications were subjected to sequencing after gene clean. The peaks were corrected from sequencing file and corrected sequences were further aligned to generate multiple alignment file by using CLUSTALW software [25]. It was observed that the representative normal (reference/wild-type) sequence was of (1-498) bp. Position '1' is marked from where the gene encoding (functional) region is started and position '498' is mentioned where the gene's encoding region is ended [25]. There was no gene mutation(s) in the control group.

Table 3. DNA Mutations

Base Insertion	Base Deletion	Base Replacement
R044 (477-478) →C	F091 (393) →A	R062 (001) T→G
R061 (191-192) →G	F091 (439) →A	
R061 (201-202) →G	F091 (468) →A	
R062 (034-035) →A	R091 (016) →A	
R103 (477-478) →C	R091 (025) →C	
	F102 (439) →A	
	F102 (478) →A	

Table 4. Frequency of Changes in DNA Bases

Sample No.	Nature of Genetic Changes	Frequency†
R44, R103	C+ (477-478)	8%
F91, F102	A- (439)	8%

†Mutation position frequency which occurred more than once. Key: Base Insertion: + Base Deletion: -.

DNA mutation analysis

Table 3 shows all DNA base changes which were identified in the radiation treated cancer patients along with position numbers. **Table 4** analyzes most frequent DNA base mutation. Frequencies are mentioned for those changes which had been occurring more than once. No DNA mutations were occurred in the control group. It is observed from **Table 4** that the most frequent (8%) DNA base alterations were found in the addition of base 'C' between positions 477-478 and in the deletion of base 'A' at position 439. All these alterations resulted into frameshift mutations. All mutations were single stranded gene mutations. Single stranded gene (hINF-α2b) mutations were also identified in leukemia as reported previously [27].

Protein translation and alignments and consequences of mutations

Protein alignments were also done in CLUSTALW software. The protein sequence alterations are discussed in Table S1 in which frameshift mutations, as a consequence of DNA base alterations are mentioned. **Table 5** describes the consequences of DNA mutations as frameshift mutations by the addition or deletion of the respective DNA base. Total 7 (32%) mutations in DNA nucleotide sequences were identified as these mutations resulted into altered protein sequences with respect to altered amino acid(s). All these mutations were frameshift mutations which is a most lethal type of mutation. There were no silent/nonsense/missense mutations reported.

It was noticed that the amino acids Serine (Ser/S) and Isoleucine (Ile/I) were found to alter twice in protein sequences at position 160 and 147 respectively.

Discussion

We have identified mutations in an anticancerous, antiviral and a defense controlling gene in cancer patients receiving radiotherapy and mentioned that how mutations identified in hINFα-2b gene sequences have led to alterations in the hINFα-2b protein. There were 32% (hIFNα-2b) gene mutations and all were frameshift mutations. Studies concerning post-radiation gene have identified single nucleotide polymorphisms (SNPs) associated with the radiation response by their influence on gene expressions [42, 43]. The results of all such studies suggested that genetic variation has an important role related to the radiation response [44]. Radiotherapy known to induce a lymphopenia and alterations in the distribution of T and B cells can result and hence these patients will compromise their immunity and there could be some hindrance in the treatment [45]. Certain cell types like 'lymphocytes' can endure apoptosis during phase of irradiation [46]. Complete blood counts (CBC) evaluation points and verified a depressed or altered immune response of cancer disease and radiotherapy treatment in cancer patients. A research group evaluated immune competence by lymphocyte-counts, delayed hypersensitivity skin tests, and in vitro response of blood lymphocytes to phytohemagglutinin of breast cancer patients who were treated with radical mastectomy. This research group [47] concluded that immunosuppression produced in the locally irradiated patients was at higher levels as compared to the postmastectomy patients who were not receiving radiotherapy [47]. The immunological state of carcinoma of the bronchus patients was assessed before and after radiotherapy in a study. Decreased immunological responses were found to correlate with shorter survival for bronchus cancer patients [48]. A conventional radiotherapy's (F×RT: radiation field during conventional radiotherapy) dose levels (threshold dose of 20 Gy) resulted in a

Table 5. Frequency of Gene Human Interferon Alpha 2b (hIFNα-2b) Mutations

Sample No.	DNA Base Position No.	Nature of Mutational Change	No. of Mutations	Frequency %
R44, R103	(C+, 477-478)	Frameshift Mutation	8 (32%)	8%
F91, F102	(A-, 439)			8%
R61	(G+, 191-192)			4%
R62	(A+, 34-35)			4%
F91	(A-, 393)			4%
R91	(A-, 16)			4%
		Nonsense Mutation	0	0%
		Missense Mutation	0	0%
		Silent Mutation	0	0%

diverse pattern of hematopoietic radiation damage [49]. A total white cell count was performed in a study to detect absorbed radiation dose effect on blood cells and detected a decrease in WBC (4.5%) and in neutrophils (12%) [50].

There is a concern that radiotherapy treated patients are on increased secondary cancer risk later in their lives [18, 51, 52]. Radiation therapy has long been linked to the incidences of secondary malignancies, including leukemia, sarcomas, thyroid, lung, and bladder carcinomas etc. [51]. A study had estimated radiotherapy induced secondary cancers in the U.K [54]. It was estimated that the risk of glioma tumors increases linearly with dose of radiation. Those who had CNS (central nervous system) tissue exposed to around 40 Gy experienced an appreciable risk. A study revealed that CNS tumors in survivors of childhood cancer indicated that the risk of meningioma increases with an increased dose of radiation to meningeal tissue. The risk of meningioma after radiation was linearly related to dose [54, 55]. A study investigated that in the treatment of prostate cancer, the risk of death from a secondary radiation-induced bladder cancer may be higher than the risk of death from the primary prostatic tumor [56]. An association between therapeutic ionizing radiation and secondary malignancies was discovered in cervical cancer patients treated with external beam radiation therapy (EBRT) [57]. Radiation-induced malignancy is the occurrence of lethal breast sarcomas following radiation treatment after lumpectomy for breast ductal carcinoma [58]. A study assessed and compared secondary cancer risk resulting from intensity-modulated radiotherapy (IMRT) and proton therapy in head-and-neck and prostate cancer patients [59]. In addition, the expression quantitative trait locus (eQTL) related studies used post-radiation, gene expression profiles have identified single nucleotide polymorphisms (SNPs) associated with radiation response through their impact on gene expression [42, 60].

Frameshift mutations (32%) in the hIFNα-2b gene are observed in radiation treated patients in this current study. It has been long reported that radiotherapy procedures result in an ample and rapid immunosuppressive response that could show a deleterious effect on the cancer patients. There have been many reports of a depressed immune response in patients with malignant disease, and also decreased levels of immunological responsive cells [48]. It is also possible that radiation induces immune-regulatory cell populations that may have relevant immune-suppressive effects, i.e., immunogenic modulation which improves tumor rejection by T cells [61]. The medical tests which determine the immunological status of cancer patients may be beneficial in monitoring the effects of radiotherapy or chemotherapy treatments [48]. The cells of the immune system are radiosensitive, hence vulnerable to the radiations. Radiation exposure induces apoptosis in mature natural killer cells, T and B lymphocytes and lethal damage in bone marrow stem cell precursors of monocytes and granulocytes [62]. Radiotherapy can considerably vary tumor-microenvironment especially the immune cells. It was hypothesized that, 'immunomodulatory cytokines' may augment the effectiveness of radiotherapy. It was found that serum concentrations of *IL-2 (Interleukin-2)* and *IFN-γ* were positively associated with the response to radiotherapy in patients with esophageal cancer. Changes in serum *IL-2* and *IFN-γ* concentrations were found associated with the increased risks of acute hematologic toxicity. Investigating the key mechanisms of radiotherapy-elicited immune response may be helpful in the production of therapeutic interventions that would enhance the adequacy of

radiotherapy following an effective immune responses [63].

Ionizing radiation is a modulator of the tumor microenvironment that has a potential for therapeutic synergy with the immune modifiers. Ionizing radiation also induces key soluble cytokines and chemokines, as well as phenotypic changes in irradiated tumor and stroma thereby further contributing to immune-mediated tumor rejection. Conversely, radiation induces expansion of immune-regulatory cell populations that may show immune-suppressive effects. There should be a comprehensive research on the use of radiation as an immune assistance because there is limited information on the induction of immune-stimulatory pathways [48]. A study [64] has examined the consequences of radiation-induced tumor cell death. Cancer cells, which die through immunogenic cell death, deliver a series of signals to the immune system that summit the generation of antitumor T cells. The ability of IR to induce an immunogenic cell death is exploited by novel cancer therapies that have shown the benefit of intra-tumoral injection of dendritic cell post-radiotherapy in preclinical models [65]. The immunological environment has been reviewed [66] which exists in tumor-bearing hosts, emphasizing the challenge to overcome the tolerance and immunosuppression to get a better tumor rejection. Combinations of IR with specific immunotherapies have been checked by several labs and observed to be effective at eliciting a strong anti-tumor immunity. One such strategy [67] used the combination of IR with intra-tumoral synthetic 'oligodeoxynucleotides' (such as *CpG*). The preclinical success of this combination was incorporated where it has demonstrated to induce rejection of the irradiated tumor as well as tumors outside the radiation field (i.e., the abscopal effect). A nanovectorized radiotherapy strategy was investigated [68] in which delivery of radionuclides using nano-particles, target specific to the tumor, was used because of the intrinsic immunostimulatory properties of nano-particles are known. However, not all IR-induced modifications of the tumor and its microenvironment can favor immune rejection [69]. A group of researchers [70] have provided a novel evidence for accumulation of pro-tumorigenic 'M2' macrophages in the area of hypoxia present in irradiated tumors. The increase of regulatory T-cells post-radiotherapy hinders the development of effective anti-tumor T-cell responses have also been discussed [71]. Another aspect addressed [72] was the dose and fractionation of radiotherapy that may modulate the expansion of effector versus regulatory T cells. Therefore, there is a need to evaluate and investigate the combination of IR and immunotherapy to improve the life of the cancer patients by immunogenic modulation strategies. The immunomodulating gene (hIFNα-2b) mutational analysis, presented here may be helpful in this connection and may guide towards specific biomarkers which correlates immunity levels and related responses with respect to the radiotherapeutical techniques and similar drugs in order to further improve a better and longer survival of cancer patients [72]. Because, the role of type-I interferons is evident as immunosurveillance and immunoediting in cancers as they are central between the interaction of tumor and the immune system [25].

The Hematopoietic system is the most radiosensitive system, it includes major blood forming system, i.e., bone marrow and circulating mature cells in the blood. Its functional cells move oxygen in the blood to provide immune protection from viruses or bacteria etc. This system ensures concentration of the blood, which preserve intact blood vessels [43, 73]. Overall, eight CBC parameters: HB, WBC, HCT, RBC, MCH, MCHC, LYM and NEUT were found below the normal range and one CBC parameter PLT was found above the normal range. Diseases like cancer can evoke high platelet count by either damage to the tissues or by the immune system that can stimulate the bone-marrow to produce more platelets. A study reported high platelet count of 6,000,000/mm^3 in patients of adenocarcinoma of the lung and refrained following radiotherapy of the primary lesion [74]. It is observed, that majority of radiation treated patients' blood parameters were suppressed from the radiotherapy treatment. However, it should be noted that these alterations may also be associated with the cancer disease they were having. However, these patients were not treated with any chemotherapeutic drugs. Decreased trend of hemoglobin, lymphocytes and neutrophils signify anemia and a depressed immune response in cancer patients. Radiotherapy treatment is known to induce a lymphopenia and hence alterations in the distribution of T and B cells can result and

hence these patients will compromise their immunity which may hinder a successful treatment course [45]. Certain cell types like lymphocytes can undergo apoptosis after irradiation [46]. Anemia is considered a common problem in many cancer patients undergoing chemotherapy or radiotherapy. Several clinical studies have indicated anemia and tumor-hypoxia in cancers. It was observed that the patients who have adequate levels of hemoglobin, will receive radiotherapy for the tumor shrink [75]. A subpopulation of stem cells, i.e., non-nucleated cells (erythrocytes and platelets) is the most resistant of the hematopoietic cells, because of their large non-cyclic state. Neutrophils and platelets which may leads to infection and bleeding. At lower doses of radiation up to 1 Gy, the white blood cell count may initially rise and then fall but usually remains within the normal range. At slightly higher doses of around 2 Gy this rise can be followed by a fall in the white blood count but depends on the amount of the dose. Neutropenia may result at higher doses of up to 8 Gy, which can raise any infection. However, the platelet count is slower to respond and usually it remains within the normal range [76]. Only 16% of cancer patients in this research reported higher levels of platelet count. The lymphocytes are the most sensitive indicators of the bone marrow injury and lymphocyte-count declines through interphase-death and rapid lysis. It was estimated in a research that 50% decline in 'absolute-lymphocyte count' occurs within a day after exposure followed by a further more severe decline within a couple of days from a potentially lethal exposure [76].

The frameshift mutations (32%) infer that the resultant protein will be produced which is a non-self protein will excite an immune response. This immunological alteration may consider a useful indicator for cancer patients receiving radiations, keeping in view the other relevant clinical inferences. Moreover, tests which determine the immunological status of patients should be conducted to monitor the effects of radiotherapy. The mechanisms of radiotherapy-elicited immune pathways may be helpful in the development of those therapeutic interventions that can enhance the profound efficacy of radiotherapy and convert ineffective responses to effective immune responses.

Summary

Human interferon alpha-2b (hIFNα-2b) gene synthesizes a protein having anticancerous and antiviral characteristics secreted by white blood cells [25]. There were 25 cancer patients treated with radiotherapeutical techniques. A suppressed lymphocytes CBC parameter is observed in radiation treated patients. Radiotherapy can substantially alter the tumor microenvironment with respect to its effects on the immune response system. The role of type-I interferons is evident as immunosurveillance and immunoediting in cancers as they are central to tumor-immune system interactions [25]. The immune controlling gene's mutational analysis presented here may be helpful in improving cancer patient's condition and longevity by analyzing those specific biomarkers which may correlate the immune levels and respective other responses with respect to the radiotherapeutical techniques and similar drugs. There were 32% frameshift mutations by which patients' bodies show significant hyper-immune response against a protein (hIFNα-2b). A depressed immunosuppression response is also reported because the immune system is radiosensitive. It is observed that the majority of the patients had their CBC parameters affected because of the ailment and radiation treatment as compared to the control group. Decreased trend of LYM, HB, MCH, MCHC and HCT signify depressed immune response and anemia in cancer patients. A radiotherapy procedure can significantly impact immune response system and cancer patients may compromise their immune system response as observed from the hematological analysis.

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Disclosure of conflict of interest

None.

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Table S1. Amino Acid Alterations in Protein Sequence as a Result of DNA Bases Change

S No.	Sample No.	Insertion of Amino Acid	Deletion of Amino Acid	Substitution of Amino Acid	Comments/Type of MUTATION
		NATURE OF CHANGE			
1	R44				FRAME SHIFTED FROM AMINO ACID Ser-S (160) TILL END AND PROTEIN SYNTHESIS TERMINATED AT POSITION 164 DUE TO STOP CODON. PROTEIN IS WITH 2 AMINO ACIDS DEFICIENCY. "FRAME SHIFT MUTATION"
2	R61	S (65-66)			FRAME SHIFTED FROM AMINO ACIDS Phe-F (64) TILL END AND PROTEIN SYNTHESIS TERMINATED AT POSITION 81 DUE TO STOP CODON. PROTEIN IS WITH 85 AMINO ACIDS DEFICIENCY. "FRAME SHIFT MUTATION"
3	R62			C→G (1)	FRAME SHIFTED FROM AMINO ACID Arg-R (12) TILL END AND PROTEIN SYNTHESIS TERMINATED AT POSITION 35 DUE TO STOP CODON. PROTEIN IS WITH 131 AMINO ACIDS DEFICIENCY. "FRAME SHIFT MUTATION"
4	F91		E (132)		FRAME SHIFTED FROM AMINO ACID 'Glu-E' FROM 132-155. "FRAME SHIFT MUTATION"
5	R91				FRAME SHIFTED FROM AMINO ACIDS Thr-T (6) TILL END AND PROTEIN SYNTHESIS TERMINATED AT POSITION 10 DUE TO STOP CODON AND RESULTANT PROTEIN IS WITH 156 AMINO ACIDS DEFICIENCY. "FRAME SHIFT MUTATION"
6	F102				FRAME SHIFTED FROM AMINO ACID Ile-I (147) TILL END AND PROTEIN SYNTHESIS TERMINATED AT POSITION 148 DUE TO STOP CODON. PROTEIN IS WITH 18 AMINO ACIDS DEFICIENCY. "FRAME SHIFT MUTATION"
7	F91				FRAME SHIFTED FROM AMINO ACID Ile-I (147) TILL END AND PROTEIN SYNTHESIS TERMINATED AT POSITION 148 DUE TO STOP CODON. PROTEIN IS WITH 18 AMINO ACIDS DEFICIENCY. "FRAME SHIFT MUTATION"
8	R103				FRAME SHIFTED FROM AMINO ACID Ser-S (160) TILL END AND PROTEIN SYNTHESIS TERMINATED AT POSITION 164 DUE TO STOP CODON. PROTEIN IS WITH 2 AMINO ACIDS DEFICIENCY. "FRAME SHIFT MUTATION"