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Deciphering the Genetic Aberrations in DNA Damage Response Genes and Their Possible Association with HNSCC

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Authors' contributions

This work was carried out in collaboration among all authors. Author JVP designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors ASSG and AP managed the analyses of the study. Author LA managed the literature searches and performed certain computational analysis. All authors read and approved the final manuscript.

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ABSTRACT

Head and neck squamous cell carcinoma (HNSCC) includes carcinomas in the oral cavity, pharynx and larynx. It is considered as the sixth most common form of cancer in the world. Several studies have confirmed that smoking and alcohol consumption are the major risk factors for HNSCC. DNA damage response genes play an important role in the maintenance of the genome. Defects in cell

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cycle checkpoint and DNA repair mechanisms, such as mutation or abnormalities, may lead to the wide spectrum of human diseases. The present study employs databases and computational tools to identify the genetic abnormalities associated with DNA damage related genes which might have a direct or indirect association with HNSCC. The demographic details of HNSCC patients was obtained from The Cancer Gene Atlas (TCGA, Firehose Legacy) dataset hosted by the cBioportal database. The oncoprint data analysis revealed the highest frequency of gene alteration in the *ATR* gene (15%), followed by *ATM, BRCA2* and *CHEK2* (5%). Other genes showed less than 5% alteration. The gene expression profile of *ATR* gene revealed its differential expression pattern in different grades of tumor relative to normal samples. The survival curve analysis using Kaplan-Meier method revealed that a high level expression of the *ATR* gene leads to poor survival rate in the female HNSCC patients when compared to males. Thus the present study has identified gross and single nucleotide variants in the *ATR* gene which could have a putative role in the development of tumor. Further experimental research is required to confirm this association.

Keywords: Carcinoma; DNA damage response genes; mutations; cell cycle checkpoints; HNSCC.

1. INTRODUCTION

Head and neck squamous cell carcinoma (HNSCC) includes carcinomas in the oral cavity, pharynx and larynx. It is considered as the sixth most cancer in the world. It's annual mortality rate was about 50%. HNSCC can be widely classified based on its location, including oral cavity, oropharynx, nasal cavity and air sinuses [1]. The factors associated with the development of HNSCC were mostly related to environmental components and viral agents. Several studies have confirmed that smoking and alcohol consumption are the major risk factors associated with HNSCC [2]. Another implicated risk factor is human papilloma virus (HPV) infection which particularly affects the oropharynx [3]. Epidemiological studies suggest that there is an increase in the incidence of oropharyngeal cancers caused by human papillomavirus [4]. DNA damage response genes play an important role in the maintenance of a healthy genome. Defects in cell cycle checkpoint or in DNA repair mechanisms, such as mutation or abnormalities, may lead to the wide spectrum of human diseases [5]. Dysregulation or mutation of DNA repair genes can affect the genomic stability, induce aging and other forms of immune deficiencies and cancer [6]. Several studies have reported that upregulation of DNA repair genes confer resistance to chemotherapy and radiotherapy [7]. The study has been reported that inhibitors of DNA pathways have the high potential to sensitize tumour inducing cells in the patient's affected with cancers [8]. Other than DNA repair pathways, these genes are also involved in nucleotide excision repair, mismatch repair, non-homologous and homologous recombination, which are found liable to all types of cancers [9]. DNA repair defects also cause various inherited defects such as aging syndromes including Ataxia telangiectasia, Nijmegen syndrome, Werner syndrome, Bloom syndrome, Xeroderma pigmentosum [10]. A study evidenced that DNA repair genes show metastasis with the onset of tumorigenesis [11]. In view of the above facts, the study has been designed to identify genetic variations which could potentiate the development of tumors.

2. MATERIALS AND METHODS

2.1 Sample Data Set

The present study follows a retrospective observational study design. The source of the patient's data was collected from the cBioportal database. This database provides an exhaustive collection of patient's details from different cohorts. The TCGA, Firehose legacy data set consisted of 528 head and neck squamous cell carcinoma cases with sequencing and number of alteration data available for 504 tumor samples [12,13]. A complete profile of mutated, amplified, deleted genes was available for each case in the dataset. The demographic details of the cases in the dataset have been provided in (Table 1). The list of twelve DNA damage response genes are available in the cBioportal database which included CHEK 1, CHEK 2, RAD 51, BRCA1, BRCA2, MLH 1, MLH2, ATM, ATR, MDC1, PARP1, FANCF. The user defined queries based on these genes returned an oncoprint data which was used for further analysis.

2.2 Oncoprint Data Analysis

The Oncoprint data analysis provided information on the type of gene alterations *viz.,* gene amplification, deep deletion, mutations/variations.

The percentage alteration and frequency of variant allele were provided for the several DNA damage response genes. These details were used to derive a putative association between the disease phenotype and genotype, to identify the variations in less understood DNA pathways or genes which could relate to HNSCC development and progression (Table 2).

2.3 Pathogenicity Analysis

PROVEAN (Protein Variation Effect Analyzer) is a computational tool used to assess the pathogenicity of a single nucleotide variant, especially the mis-sense type. The reference protein sequences of all the genes encoding proteins selected in the study were obtained from the NCBI database (https://www.ncbi.nlm.nih.gov/protein). Based on the scores obtained the pathogenicity of the variant was determined (Table 3). A score less than -2.5 or greater than -2.5 considered to be as deleterious and neutral respectively [14].

2.4 Gene Expression and Survival Curve Analysis

The expression of the gene in HNSCC was analysed using the UALCAN (http://ualcan.path.uab.edu/cgi-bin/TCGA-survival1.pl?genenam=)database. Survival curve analysis based on the gender and expression profile was performed to demonstrate the putative role of *ATR* gene with HNSCC. Gene expression data is expressed as transcripts per million (TPM). Kaplan-Meier survival analysis was performed to identify the prognostic value of the gene with highest frequency of genetic alteration [15].

3. RESULTS AND DISCUSSION

Genomic instability is the significant feature of cancer cells. It is caused by down regulation of the DNA damage response pathways which is controlled by *ATM*, *Rad3* and *ATR* genes [16]. Many studies have performed systematic analysis on various types of DNA damage response genes. The *RAD9* gene functions as part of a heterotrimer with *RAD1* gene, which is involved in apoptosis and controller of cell cycle checkpoints in DNA damage pathways [17,18]. The present study was intended to identify genetic alterations in DNA damage genes in HNSCC patients. The frequency of genetic alterations ranged from 0.4 to 15%. Among the various DNA damage response genes, the *ATR* gene showed the highest frequency (15%) of gene alteration. The frequency of genetic alteration in other genes were as follows: *CHEK 1* (1.2%), *CHEK 2* (5%), *RAD51* (0.4%), *BRCA1* (2.6%), *BRCA2* (5%), *MLH1* (1.8%), *MLH2* (1.6%), *ATM* (5%), *PARP1* (2%), *FANCF* (1%) and *MDC1* (4%) (Fig. 1; Table 2). Gene amplification and mutations

Table 1. Demographic details of patients analysed in the present study (as obtained from the cBioportal site)

Gender	Male (n = 386)
	Female (n = 142)
Mutation count	6-3181
Diagnosis age	19-90 years
Smoking status	Smokers: 515
	Data not available: 12
	Unknown: 1
Alcohol history	Yes – 352
	No – 165
	Data not available: 11
Neoplasm Histologic grade	Grade 1: 63
	Grade 2: 311
	Grade 3: 125
	Grade 4: 7
	Grade GX: 18
	Data not available: 4
Race category	White: 452
	African: 48
	Asian: 11
	American Indian or Alaska native: 2
	Data not available: 15

Table 2. The frequency of genetic alterations, cytogenetic location of the gene, protein encoded by genes, variant allele frequency in tumor sample for the DNA damage response family of genes

Gene	Protein	Alteration	Cytogenetic location	% of alteration	Variant allele frequency in tumor sample
CHEK1	Checkpoint Kinase 1	Gene Amplification	11q24.2	1.2	
		Deep Deletion			
		R379*			
					0.1
CHEK2	Checkpoint Kinase 2	Gene Amplification	22q12.1	1.8	
		T323Lfs*14			0.45
		N166S			0.07
		T532I			0.21
		S372F			0.26
RAD51	RAD51 Recombinase	Gene Amplification	15q15.1	0.6	
		Deep Deletion			0.45
		S296L			
BRCA1	BRCA1 DNA Repair Associated	Gene Amplification	17q21.31	2.6	
		X101_Splice			0.37
		D853N			0.27
		H816Y			0.05
		G1492R			0.23
		E230Q			0.38
		D1505N			0.22
		R664G			0.14
		R1645M			0.47
		R1737T			0.12
		S184C			0.04
		K175N			0.14
BRCA2	BRCA 2 DNA Repair Associated	Deep deletion	13q13.1	4	
		Y2884*			0.20
		R2625*			0.26
		Q2749*			0.35
		Y839Ifs*42			0.10
		E2175Q			0.25

Gene	Protein	Alteration	Cytogenetic location	% of alteration	Variant allele frequency in tumor sample
		E3342K			0.27
		G500V			0.43
		V1605D			0.05
		E1571K			0.60
		I2105V			0.44
		E2903K			0.16
		P606L			0.13
		K3315N			0.38
		S3218F			0.07
		R2861T			0.25
		F312C			0.33
		S3231L			0.23
		D1386N			0.27
		E3393Q			0.18
		S3123G			0.13
		P1039H			0.13
MLH1	MutL Homolog 1	Deep deletion	3p22.2	1.4	
		E102*			0.56
		1262M			0.28
		A281V			0.07
		P138R			0.43
		V647L			0.13
MLH2	DNA Mismatch repair protein Msh2	Deep deletion	2q32.2	1.8	
		A230Lfs*16			0.08
		G162V			0.23
		R382C			0.19
		A107T			0.29
		S142L			0.13
		A434V			0.16
		Q314R			0.27
ATM	ATM Serine/Threonine Kinase	Gene Amplification	11q22.3	5	
		Deep Deletion			0.77
		R337C			0.18

Gene	Protein	Alteration	Cytogenetic location	% of alteration	Variant allele frequency in tumor sample
		X1726_splice			0.20
		X25_splice			0.42
		12899M			0.27
		Y1248C			0.22
		D1053N			0.30
		1238V			0.28
		I1035V			0.16
		W412C			0.54
		K2749N			0.39
		D2997N			0.28
		E2932K			0.07
		D2988N			0.11
		V1506M			0.12
		E2238Q			0.33
		T2333I			0.24
		T2754A			0.11
		H636N			
ATR	ATR Serine/Threonine Kinase	Gene Amplification	3q23	10	
		Y2637			0.14
		W1800R			0.35
		S1348F			0.10
		M2266I			0.15
		S61C			0.10
		H1684D			0.07
		E2373V			0.19
		E560K			0.12
		P2549T			0.51
		G2319E			0.25
		I693M			0.10
		E54Q			0.06
		N2199S			0.27
		V66M			0.16
		S549C			0.40

Gene	Protein	Alteration	Cytogenetic location	% of alteration	Variant allele frequency in tumor sample
		F99L			0.27
		S246F			0.10
		L400F			0.06
		L987I			0.17
		R215S			0.09
		V1802A			0.13
		D1243H			0.10
MDC 1	Mediator Of DNA Damage Checkpoint 1	Gene Amplification	6p21.33	4	
		E459K			0.21
		S1253Y			0.19
		S1743C			0.21
		D1175Y			0.29
		L122F			0.71
		D1845N			0.28
		S1440F			0.05
		R114H			0.28
		D134V			0.25
		Q1056*			0.14
		H354Y			0.14
		N1397I			0.28
		G1401C			0.08
		H368N			0.11
PARP1	Poly(ADP-Ribose) Polymerase 1	Gene Amplification	1q42.12	1.4	
		S274F			0.23
		E456Q			0.27
		P174T			0.54
FANCF	FA Complementation Group F	L86F	11p14.3	0.4	0.33
		A250D			0.29

(truncating and non-synonymous) were found in the ATR gene. The PROVEAN predictions of genes reported both neutral and deleterious outcomes across the *CHEK2, RAD 51, BRCA1, BRCA2, ATR, ATM* group of DNA damage response genes (Table 3).

The gene expression profile observed in HNSCC patients could be related to the gene amplification documented earlier (Fig. 2). The comparison of gene expression patterns between different grades of HNSC returned significant values between normal and different grades of tumor viz., grade 1, grade 2 grade 3. Significant difference in gene expression was observed between different grades also (Fig. 3). Furthermore, the survival probability analysis employing Kaplan–Meier plots showed significant association of *ART* gene expression in combination with the gender with HNSCC patient's survival. The high level expression of the *ATR* gene in females in comparison to male patients provided a significant result with a p value = $<10^{-12}$. The differential gene expression pattern and the associated survival probability in male and female HNSCC patients is suggestive of the prognostic significance of the gene (Fig. 3).

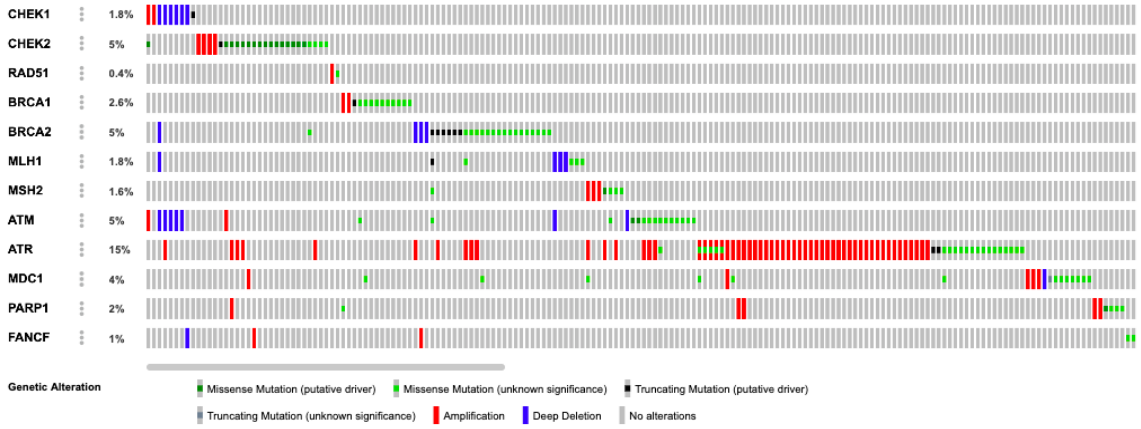

Fig. 1. On coprint data depicting gene alterations in the DNA damage response genes. Each of the grey bars represent HNSCC patients

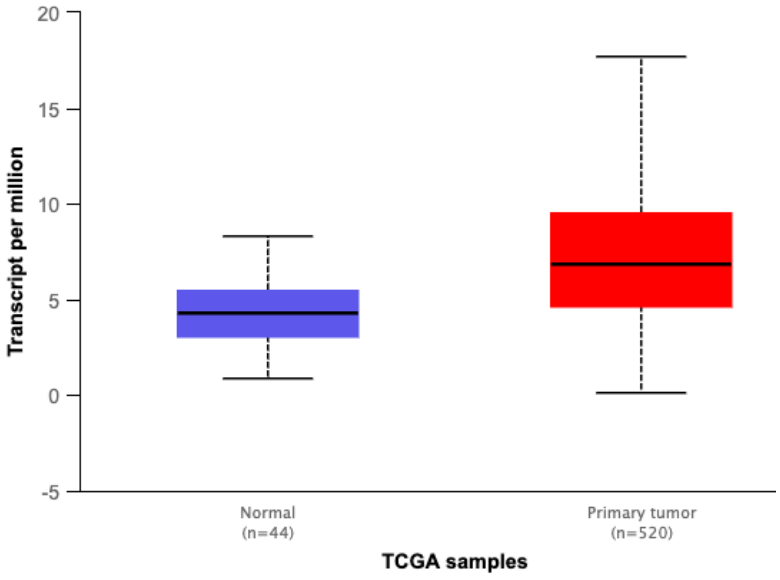

Fig. 2. Box-Whisker plot showing relative expression profile of *ART* gene in primary tumor of HNSC patients in comparison to normal samples (p value = $<10^{-12}$. The X axis denotes the TCGA samples and Y axis denotes the transcripts per million values. A p value less than 0.05 is considered to be significant

Table 3. Pathogenicity of missense variants identified in DNA damage response genes as predicted by PROVEAN

Gene	Alteration	PROVEAN Score	PROVEAN Prediction
CHEK2	N166S	-4.818	Deleterious
	T532I	-0.767	Neutral
	S372F	-5.551	Deleterious
RAD51	S296L	-5.658	Deleterious
BRCA1	D853N	-4.562	Deleterious
	H816Y	-4.582	Deleterious
	G1492R	-1.206	Neutral
	E230Q	-0.553	Deleterious
	D1505N	-0.312	Neutral
	R664G	-5.388	Deleterious
	R1645M	-0.520	Neutral
	R1737T	-1.067	Neutral
	S184C	-0.663	Neutral
	K175N	0.289	Neutral
BRCA2	E2175Q	-0.536	Neutral
	E3342K	-1.161	Neutral
	G500V	-1.570	Neutral
	V1605D	-5.366	Deleterious
	E1571K	-3.041	Deleterious
	I2105V	-0.132	Neutral
	E2903K	-0.965	Neutral
	P606L	-0.928	Neutral
	K3315N	-0.859	Neutral
	S3218F	-1.141	Neutral
	R2861T	-1.187	Neutral
	F312C	-1.747	Neutral
	S3231L	-1.571	Neutral
	D1386N	-4.722	Deleterious
	E3393Q	-0.493	Neutral
	S3123G	-1.161	Neutral
	P1039H	-6.775	Deleterious
MLH1	I262M	-2.815	Deleterious
	A281V	-2.945	Deleterious
	P138R	-3.415	Deleterious
	V647L	-1.167	Neutral
MLH2	G162V	-8.287	Deleterious
	R382C	-6.674	Deleterious
	A107T	-0.789	Neutral
	S142L	-3.304	Deleterious
	A434V	-1.884	Neutral
	Q314R	-1.502	Neutral
ATM	I289M	-1.232	Neutral
	Y1248C	-5.480	Deleterious
	D1053N	-2.808	Deleterious
	I238V	-0.273	Neutral
	I1035V	0.447	Neutral
	W412C	-6.890	Deleterious
	K2749N	-2.848	Deleterious
	D2997N	-1.090	Neutral
	E2932K	-3.314	Deleterious
	D2988N	-0.849	Neutral
	V1506M	-0.786	Neutral
	E2238Q	-0.655	Neutral

Gene	Alteration	PROVEAN Score	PROVEAN Prediction
	T2333I	1.474	Neutral
	T2754A	-3.037	Deleterious
	H636N	-1.195	Neutral
ATR	Y2637C	-8.160	Deleterious
	W1800R	-8.567	Deleterious
	S1348F	-2.818	Deleterious
	M2266I	-1.767	Neutral
	S61C	-1.715	Neutral
	H1684D	0.877	Neutral
	E2373V	-6.183	Deleterious
	E560K	-0.457	Neutral
	P2549T	0.185	Neutral
	G2319E	-7.600	Deleterious
	I693M	-0.161	Neutral
	E54Q	-0.784	Neutral
	N2199S	-1.026	Neutral
	V66M	-0.518	Neutral
	S549C	-1.204	Neutral
	F99L	-1.518	Neutral
	S246F	-0.880	Neutral
	L400F	-1.426	Neutral
	L987I	-0.402	Neutral
	R215S	-0.670	Deleterious
	V1802A	-2.675	Deleterious
	D1243H	-2.123	Neutral
MDC 1	E459K	-2.178	Neutral
	S1253Y	-2.167	Neutral
	S1743C	-3.572	Deleterious
	D1175Y	-3.250	Deleterious
	L122F	-2.333	Neutral
	D1845N	-1.256	Neutral
	S1440F	-3.550	Deleterious
	R114H	-2.650	Deleterious
	D134V	-3.683	Deleterious
	H354Y	-2.667	Deleterious
	N1397I	-2.783	Deleterious
	G1401C	-1.226	Neutral
	H368N	3.383	Deleterious
PARP1	S274F	-3.825	Deleterious
	E456Q	-2.167	Neutral
	P174T	-0.649	Neutral
FANCF	L86F	-2.729	Deleterious
	A250D	-2.529	Deleterious

The *ATR* gene (OMIM 601215) encodes the protein Ataxia telangiectasia and Rad3-related protein, which is a serine-threonine kinase. It senses DNA damage and activates cell cycle checkpoints when induced by endogenous stressors. The protein phosphorylates and activates several other proteins involved in the inhibition of DNA replication and cell division providing time for the cell to repair its DNA, promote recombination and apoptosis [19]. The *ATR* gene acts along with its closest relative *ATM* (Ataxia Telangiectasia Mutated). Both the proteins work in consonance and activate the major regulator of stress or damage response gene p53 [20]. The p53 gene is considered to be the “guardian of the genome” and is often inactivated by loss of function mutations in several cancer types over 50%. The frequency of mutations or gene alterations observed in p53 is reported to be high i.e., over 70% in HNSCC [21].

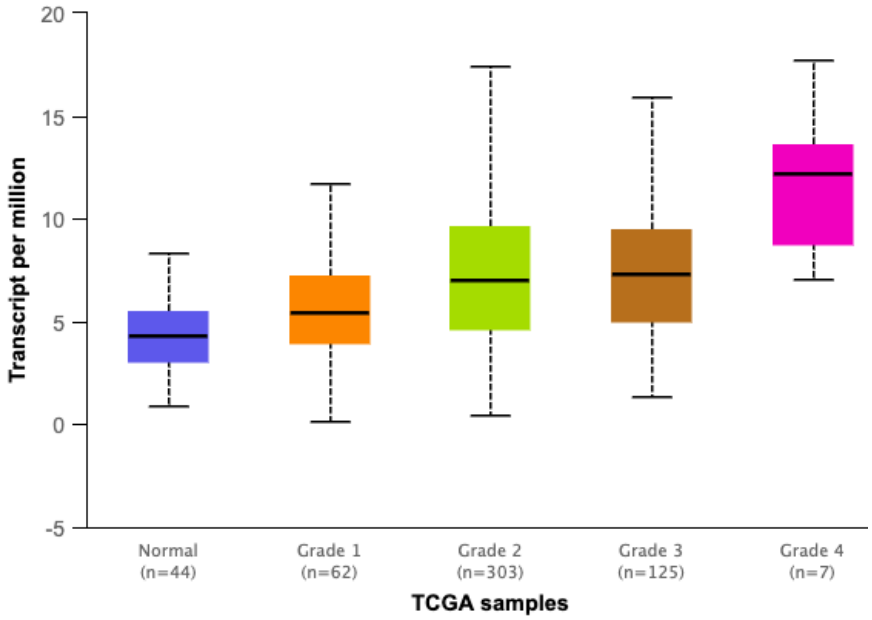

Fig. 3. Box-Whisker plot showing relative expression profile of *ATR* gene in different grades of HNSC. The X axis denotes the TCGA samples and Y axis denotes the transcripts per million values. The comparison of gene expression patterns between different grades of HNSC returned significant values between normal vs grade 1 ($p=6.4 \times 10^{-4}$), normal vs grade 2 ($p < 10^{-12}$), normal vs grade 3 ($p=2.6 \times 10^{-14}$), normal vs grade 4 ($p=1.9 \times 10^{-3}$), grade 1 vs grade 2 ($p=4.5 \times 10^{-4}$), grade 1 vs grade 3 ($p=3.6 \times 10^{-3}$), grade 1 vs grade 4 ($p=4.03 \times 10^{-4}$) and grade 3 vs grade 4 ($p=1.56 \times 10^{-2}$). A p value less than 0.05 is considered to be significant

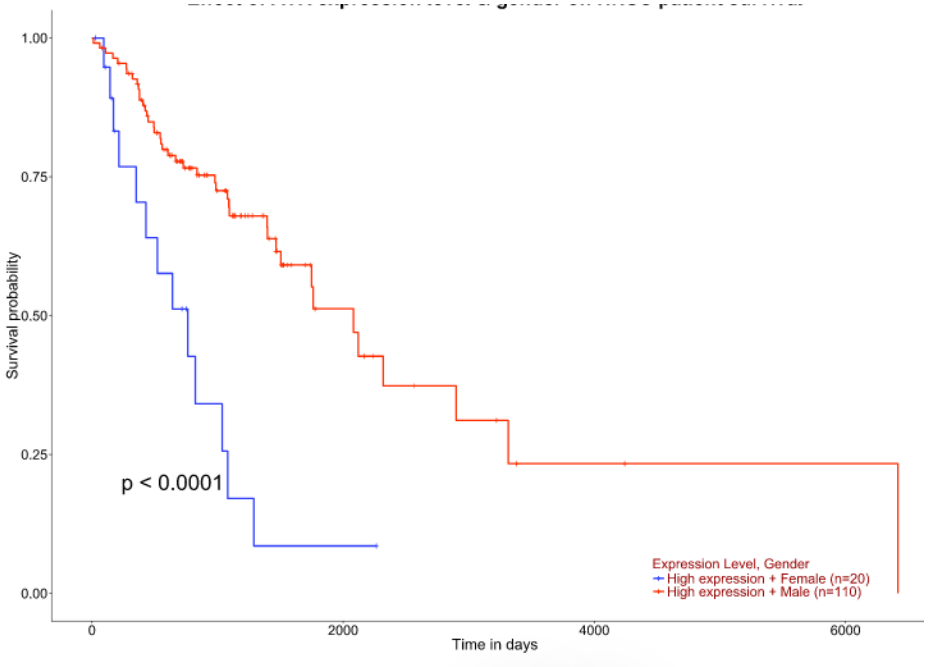

Fig. 4. Kaplan–Meier plots showing the association of high level expression of *ATR* gene in male and female HNSC patient's survival. The x-axis represents time in days and y-axis shows the survival probability. The blue line indicates high level expression in female patients and the red line indicates high level expression in male patients. A high level expression of the *ATR* gene presented with a low survival rate in female patients (p value < 0.0001)

The *ATR* gene mutations have been reported in melanoma, lung, stomach, bladder, uterine and endometrial cancers with less than 2% alteration in HNSCC. The present study identified less than 5% mutations in the *ATR* gene. Although germline mutations in *ATR* gene are rare events, such mutations have been identified in patients with Seckel syndrome, who present with an autosomal dominant type of inheritance [22]. A study reported novel mutations in ATR gene in oropharyngeal squamous cell carcinoma (OpSCC) patients, with an HPV negative status. They concluded that the mutations identified could lead to functional loss of the gene activity thereby indicating the important role of the gene in the etiology of OpSCC [23]. Another study by Parikh et al., demonstrated the upregulation of ATR-CHEK1 pathway in OSCC cell lines with the loss of ATM. They observed gene amplification, translocations and gain of copy number in ATR gene. The qPCR assay also showed overexpression of ATR in OSCC cell lines. The inhibition of ATR with siRNA was found to increase the sensitivity of OSCC cells to ionizing radiations [24]. A very recent study by Farah and colleagues, reported the association of FA/BRCA of the double stranded break pathway (DSB) with the malignant transformation of oral leukoplakia to oral squamous cell carcinoma (OSCC) [25].

The ATR inhibitors have been well documented using *in vitro* approaches. A recent study demonstrated the sensitisation of HNSCC cell lines to cisplatin upon inhibition of ATR with AZD6738. The results were also replicated successfully in patient-derived xenograft tumors. The HPV negative and HPV positive cell lines were also found to be sensitive to cisplatin by inhibition of ATR [26]. A critical determinant of sensitivity to radiotherapy in patients with oropharyngeal cancer with HPV positive patients has been linked to defects in signal mediators and repair genes. The principal target proteins were PARP; DNA-Pk, ATM and ATR. Also inhibitors of proteins involved in cell cycle checkpoint activation were also tested for radiosensitization of HNSCC [27]. Another interesting finding about the role of ATM and ATR in relation to HPV DNA replication was recently documented. The inhibition of ATR pathway resulted in the suppression of HPV amplification and maintenance of the genome. The underlying mechanisms could be through molecular pathways such as transcriptional regulation and cell cycle checkpoints [28,29]. Thus, the present study provides preliminary details on the genetic alterations in ATR-ATM pathway and its possible involvement in the development of HNSCC. The *in silico* approaches have been found to be successful in screening for alterations in candidate gene family in association with HNSCC [30]. DNA damage response gene expression levels significantly correlated with the tumor grades in HNSCC patients. The defects in cell cycle checkpoints of DNA repair genes, such as mutation or abnormalities, may lead to the wide spectrum of human diseases.

4. CONCLUSION

The present study thus highlights the mutations in DNA damage response genes which may have a significant role to play in HNSCC. Further experimental validations are warranted to derive an association between the preliminary data obtained and the disease phenotype.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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