Mutagenesis vol. 28 no. 2 pp. 213–218, 2013 doi:10.1093/mutage/ges074

Genetic and expressional variations of *APEX1* are associated with increased risk of head and neck cancer

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Received on May 30, 2012; revised on October 24, 2012; accepted on November 13, 2012

The aetiology of head and neck cancer (HNC) has been shown to be associated with genetic and certain environmental factors that produce DNA damage. Base excision repair (BER) genes are responsible for repair of DNA damage caused by reactive oxygen species and other electrophiles and therefore are good candidate susceptibility genes for HNC. Apurinic/apyrimidinic endonuclease-1 (APEX1) proteins have important functions in the BER pathway. In this case–control study, all exons of the *APEX1* gene and its exon/intron boundaries were amplified in 300 HNC cases and 300 matched healthy controls and then analysed by single-stranded conformational polymorphism. Amplified products showing altered mobility patterns were sequenced and analysed. To confirm our observations, we examined *APEX1* expression at mRNA level on 50 head and neck squamous cell carcinoma (HNSCC) and 50 normal control samples by quantitative real-time polymerase chain reaction. At germ line level, three novel mutations (13T > G, Ser129Arg and Val131Gly) of APEX1 were observed. The homozygous and heterozygous genotypes of APEX1 13T > G, Ser129Arg and Val131Gly appear to be significantly involved in the development of HNC. In the case of expressional level, *APEX1* mRNA expression was positively correlated with tumour size, clinical stage and positive lymph node metastasis. Statistical analysis showed a significantly higher *APEX1* mRNA level in HNC tumour tissue than in control samples. Our study demonstrated that *APEX1* mutations and deregulation of *APEX1* are associated with increased risk of HNC in the Pakistani population.

Introduction

Head and neck cancer (HNC) is not a specific entity, but rather a broad category of diverse tumour types arising from various anatomical structures including the craniofacial bones, soft tissues, salivary glands, skin and mucosal membranes (1). HNC is the sixth most common cancer worldwide accounting for 4% of cancer in men and 2% of cancer in women (2). In some parts of the world including southern China, Pakistan, India, Thailand and Brazil, HNC is a major cancer problem (3).

Head and neck carcinogenesis is associated with abnormalities in DNA repair, apoptosis, carcinogen metabolism and cell-cycle control (4). DNA repair systems play a critical role in protecting the human genome from damage caused by carcinogens present in the environment (5). Apurinic/apyrimidininc endonuclease 1 (APEX1) protein has important functions in the base excision repair (BER) pathway (6). *APEX1* resides on 14q11.2–14q12 and processes the AP sites or single-strand breaks (SSBs) remaining after the damaged base has been excised by DNA glycosylases, considered the rate-limiting step in BER (7). APEX1 has two domains, a DNA-repairing domain and a redox domain. The carboxy-terminus of APEX1 contains the endonuclease activity required for DNA repair and spans residues 61–318, whereas residues 1–127 comprise the redox domain (8).

APEX1 has been characterised in numerous functional studies. The *Escherichia coli* APEX1 homologue xthA demonstrated hypersensitivity to hydrogen peroxide and ultra violet light when AP activity was eliminated (9,10) and yeast defective in AP site repair exhibited an elevated spontaneous mutator phenotype (11). Ramana *et al.* (12) demonstrated that human cells exposed to sublethal doses of oxidising agents showed an increase in both the amount of APEX1 and APEX1 activity. *APEX1* has also been shown to interact with X-ray repair cross-complementing protein 1 (XRCC1) (13) and *APEX1* over-expression can compensate for *XRCC1*-deficient cells in the repair of DNA SSBs induced by oxidative DNA damage, both *in vivo* and in whole-cell extracts (14).

The limited epidemiological data examining the relationship between *APEX1* polymorphisms and cancers and related traits have suggested a weak to null effect, although multiple studies of the same outcome are few (15–17). Many investigators have focused on the Asp148Glu variant, as it resides in the carboxy-terminus. However, variants outside the *APEX1* DNA repair domain, such as residues 61–318 or promoter regions, could be markers for the disease-causing single nucleotide polymorphism (SNP) and may be informative (18).

In this work, we have studied if the polymorphisms/mutations in the *APEX1* gene are able to modify the risk for HNC, and if the effects of these polymorphisms/mutations differ in never-smokers and smokers. In addition to this, we also measured mRNA levels of *APEX1* in patients with HNC. The main focus was to investigate any mutation or expressional change in the *APEX1* gene in HNC in a Pakistani population.

Materials and methods

The present population-based case–control study was conducted with a prior approval from ethical committees of both COMSATS Institute of Information Technology and collaborating hospitals. Two HNC patient groups were recruited in this study. Clinical characterisation of the patients is summarised in Table I. The cohort 1 was used for evaluating the *APEX1* variants at germ line level. This group consists of blood of 300 HNC cases and 300 matching normal healthy individuals as controls. The selection procedure for patients included confirmed histological diagnosis of HNC, no preoperative therapy and availability of complete follow-up data. The inclusion criterion for the controls was absence of prior history of cancer or precancerous lesions. Patients and controls suffering from any other familial disease (diabetes, blood pressure and cardiovascular impairment) were excluded from this study. After obtaining informed consent, all individuals were personally interviewed using the specifically designed questionnaire. Information on age, gender, ethnic group and detailed exposure data on smoking was recorded. This study included three types of tobacco use, which are betel quid, moist sniff and cigarettes. As intake of these agents varies from individual to individual, a broad definition of smoking was introduced here.

Table I. Demographic and clinical characterisation of the cohorts

Variables		Cases	Controls
Cohort 1			
Age	Median (range)	55 (25–80)	57 (25–80)
Gender	Male: *n* (%)	152 (51)	156 (52)
	Female: *n* (%)	148 (49)	144 (48)
Family history	Yes: *n* (%)	25 (8)	4 (1)
	No: *n* (%)	275 (92)	296 (99)
Smoking	Smokers: *n* (%)	129 (43)	140 (47)
	Non-smokers: *n* (%)	171 (57)	160 (53)
Cohort 2[a]			
Age	Median (range)	60 (20–80)	60 (20–80)
Gender	Male: *n* (%)	27 (54)	27 (54)
	Female: *n* (%)	23 (46)	23 (46)
Clinical stage	Stage I: *n* (%)	05 (10)	–
	Stage II: *n* (%)	09 (18)	–
	Stage III: *n* (%)	26 (52)	–
	Stage IV: *n* (%)	10 (20)	–
T stage	Stage I: *n* (%)	05 (10)	–
	Stage II: *n* (%)	11 (22)	–
	Stage III: *n* (%)	28 (56)	–
	Stage IV: *n* (%)	06 (12)	–
N stage	Stage 0: *n* (%)	16 (32)	–
	Stage I: *n* (%)	28 (56)	–
	Stage II: *n* (%)	06 (12)	–
Grade	Poor: *n* (%)	05 (10)	–
	Mod: *n* (%)	15 (20)	–
	Well: *n* (%)	30 (60)	–

[a]M stage data for HNSCC patients are not available.

Cohort 2 was used for evaluating *APEX1* expression at mRNA level. This group included tumours of 50 HNC patients who underwent surgery at Military Hospital (Rawalpindi, Pakistan) between 2009 and 2012. Clinical, epidemiological and histopathological characteristics of these patients are shown in Table I. All tumours are histologically confirmed to be squamous cell carcinoma of HNC and were graded as well differentiated, moderately differentiated and poorly differentiated. The tumour extent was classified according to the tumour, lymph node, metastasis (TNM) staging system by International Union Against Cancer (UICC) (19). Among 50 cases, 34 (68%) had histologically confirmed lymph node metastasis (N+), whereas remaining 16 (32%) had no lymph node metastasis (N0). Samples of tumour core, the invasive edge of tumour and microscopically healthy mucosa (control) were obtained from each surgical section. Presence of tumour cells in the collected tissues was rectified by examination of frozen sections following haematoxylin and eosin stain by a consultant pathologist. Whereas samples of control mucosa were obtained from macroscopically confirmed uninvolved healthy area >2 cm away from the tumour.

DNA extraction and polymerase chain reaction

DNA was extracted from white blood cells of study group 1, using standard phenol–chloroform extraction method (20) and stored at −20°C for further processing. Human *APEX1* exon sequence was taken from Ensembl. Primers were designed by using primer 3 software, and checked for specific amplification using BLAST. Whole coding region and its exon/intron boundaries of ~60 bp sequence of *APEX1* were investigated to identify any splice site variation. Each polymerase chain reaction (PCR) was performed in a 20 µl reaction mixture containing ~20 ng of genomic DNA templates, 2 µl (10 mM) of each primer, 0.24 µl (25 mM) of dNTP, 2 µl (10×) PCR buffer and 0.2 µl (5 u/µl) of *Taq* polymerase. PCR profile consisted of an initial melting step of 94°C for 5 min, 35 cycles of 94°C for 45 s, annealing temperature for 1 min and 72°C for 1 min and a final extension step of 72°C for 10 min followed by hold at 4°C. PCR products were electrophoresed on a 2% agarose gel and stained with ethidium bromide.

Mutational screening and sequencing

Single-stranded conformational polymorphism (SSCP) was used for the mutational analysis of the PCR products. Samples, displaying an altered electrophoretic mobility, were reamplified in another reaction and were analysed by direct sequencing to confirm and characterise the nature of mutations. Sequencing was carried out by MCLab (USA). Control (normal) samples were also sequenced along with cases to check the quality of sequencing.

RNA extraction and real-time PCR

RNA isolation was carried out from tumour samples of study group 2 by using the standard Trizol reagent method (21) and stored at −80°C. Reverse transcription PCR (RT–PCR) was carried out using SuperScript First-Strand Synthesis System (Invitrogen, USA). The SuperScript III First-Strand Synthesis System for RT–PCR was optimised to synthesise first-strand cDNA from purified poly(A)$^+$ or total RNA. It provides high cDNA yields, sensitivity and specificity.

For quantitative PCR (qPCR), primers specific for gene *APEX1* and *β actin* (internal control) were purchased from OriGene (USA). Each qPCR was performed in a 20 µl reaction mixture containing ~1 µl of product from RT reaction, 10 µl of 2× Sybr Green, 1 µl of each primer and 7 µl RNase free water. qPCR was performed using Real-Time PCR system (Applied Biosystems Step one plus) under standard conditions.

The relative mRNA levels of *APEX1* and *β actin* were computed using the $2^{-\Delta\Delta Ct}$ analysis method (22).

Structure prediction

Wild-type protein model was obtained from protein data bank. Mutated structure of APEX1 was predicted using automated Swiss model. Two structures, wild and mutated, were aligned using UCSF chimera software.

Statistical analysis

For each mutation, deviation of the genotype frequencies in the control subjects from those expected under Hardy–Weinberg equilibrium was calculated. We estimated the cancer risk associated with the alleles, genotypes as odds ratios (ORs) and 95% confidence intervals (CIs) by using unconditional logistic regression with adjustment for age, sex and smoking status in study group 1. *P*-values for trend were calculated by chi-square test.

For study group 2, one-way analysis of variance was used to assess the association of *APEX1* expression with clinical and histopathological parameters (e.g. TNM and grade). Spearman correlation coefficient was used to assess correlations among the gene expression and clinical and histopathological parameters.

Results

Mutation screening

Three *APEX1* variants were identified in patients and controls after SSCP followed by the sequencing in study group 1 (Figure 1). One substitution (13T < G) mutation was found in the 3′UTR region and two non-synonymous mutations (Ser129Arg, Val131Gly) are found in the fourth exon of the gene *APEX1*. Frequency of all mutations showed significant difference between HNC patients and control subjects. The genotype frequency of these *APEX1* mutations in cases and controls is shown in Table II. In case of 13T < G mutation, ~5-fold increase in HNC risk (OR = 4.79, 95% CI = 2.36–10.44) was associated with GG genotype (major homozygote) and ~2.0-fold increase (OR = 1.83, 95% CI = 0.91–3.69) with GT genotype (heterozygote). For the Ser129Arg mutations, ~12-fold increase in HNC risk (OR = 11.74, 95% CI = 4.98–27.68) was associated with GG genotype (major homozygote) and ~6.0-fold increase (OR = 6.31, 95% CI = 2.40–16.54) with GT genotype (heterozygote). In case of Val131Gly, ~5.0-fold increase in HNC risk (OR = 5.21, 95% CI = 2.61–10.07) was associated with GG (major homozygote) and ~3.0-fold increase (OR = 2.59, 95% CI = 1.38–4.84) with GT genotype (heterozygote). These risks persisted even when the data were adjusted for chi-square analysis and was statistically significant.

The largest transcript of APEX1 consists of 318 amino acids making six identical chains as seen in its structure (Figure 2). Protein modeling of two mutations (Ser129Arg, Val131Gly) of *APEX1* and comparison with wild-type APEX1 protein has concluded that no major change occurs with these mutations.

Analysis of gene–environment interaction

We observed a significant interaction between smoking and mutations in the selected gene, *APEX1*. We noticed that the smokers with the 13T < G-GG genotype had higher risk of HNC than GT genotype and smokers with Ser129Arg-GG and GT genotypes had equal incidence of HNC. Smokers with the Val131Gly-GG

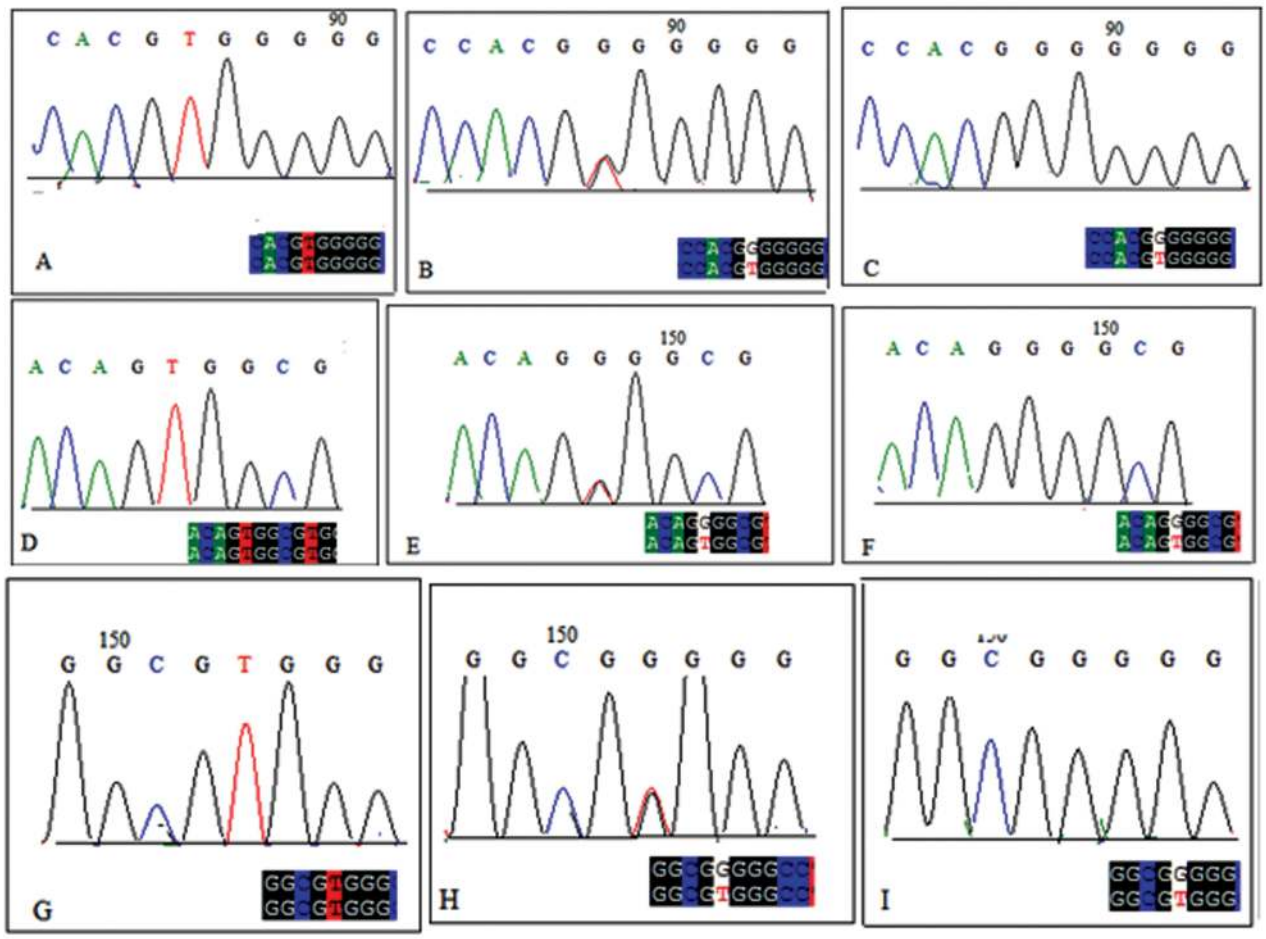

Fig. 1. Nucleotide sequence of fragments of *APEX1*. (**A**) Wild-type codon, (**B**) mutant heterozygous codon, (**C**) mutant homozygous codon of 3′UTR, 13T < G. (**D**) Wild-type codon, (**E**) mutant heterozygous codon, (**F**) mutant homozygous codon of missense mutation Ser129Arg, showing T to G substitution resulting in change of DNA sequence from AGT to AGG encoding the amino acid Arginine instead of Serine. (**G**) Wild-type codon, (**H**) mutant heterozygous codon, (**I**) mutant homozygous codon of missense mutation, Val131Gly showing T to G substitution resulting in change of DNA sequence from GTG to GGG encoding the amino acid glycine instead valine.

genotypes had higher risk of HNC than GT genotype, similar trend was observed for these genotypes in non-smoker HNC cases when compared with control subjects (Table III).

The APEX1 mRNA level in HNSCC

APEX1 mRNA level was observed in 50 HNC tumour and 50 normal tissue samples in study group 2. *APEX1* expression is significantly higher in HNC tumour than in normal tissue samples. Statistical significant increase in *APEX1* mRNA level was observed in the tissues with positive lymph node metastasis (pN+) as compared with those with negative status. Increased *APEX1* expression was also observed in larger (T3–T4) tumour tissues as compared with smaller (T1–T2) tumours. The expression level of *APEX1* is higher in late stage (III–IV) than in early stage disease (I–II). In case of tumour grades, somewhat higher expression was observed in well-differentiated tumour than in poor to moderately differentiated tumours, but this increase is not statistically significant (Figure 3).

Table II. Allele and genotype frequencies of *APEX1* gene and their associations with risks for HNC

Mutations/exons	Series	Minor allele frequency	No. of major homozygote TT	No. of heterozygote GT	No. of minor homozygote GG	*P-value
13T < G	Case patients	0.17	237	23	40	0.0001
3′UTR	Control subjects	0.05	278	13	09	
	[a]OR (95% CI)		1.00 (ref)	1.83 (0.91–3.69)	4.97 (2.36–10.44)	
Ser129Arg	Case patients	0.24	213	36	48	0.0001
Exon 4	Control subjects	0.02	289	15	06	
	[a]OR (95% CI)		1.00 (ref)	6.31 (2.40–16.54)	11.74 (4.98–27.68)	
Val131Gly	Case patients	0.22	215	36	49	0.0001
Exon 4	Control subjects	0.05	274	15	11	
	[a]OR (95%CI)		1.00 (ref)	2.59 (1.38–4.84)	5.12 (2.61–10.07)	

[a]ORs were adjusted by age, sex and smoking status.
*$P > 0.05$, by chi-square test for trend.

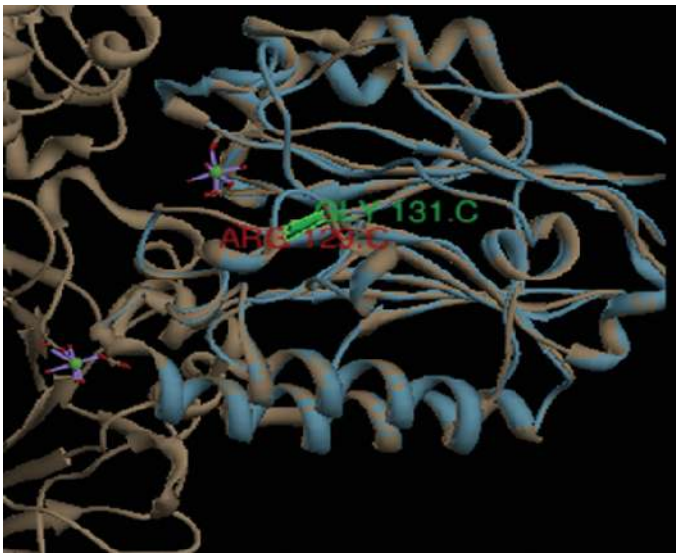

Fig. 2. Superimposed protein structure of mutated APEX1 with its wild type. Wild-type APEX1 protein (grey) and mutated APEX1 protein (blue). Superimposed protein structure of mutated APEX1 showing the location of two observed mutations, Ser129Arg (red) and Val131Gly (green). Wild-type protein model obtained from protein data bank. Structure was predicted using automated Swiss model. Two structures, wild and mutated, were aligned using UCSF chimera software.

Correlation between APEX1 expression and clinicopathological characteristics

Correlations were tested between *APEX1* expression, clinical and pathological features of HNC patients group 1. The *APEX1* mRNA level was positively correlated with T stage, C stage and pN stage. pT stage was positively correlated with C stage, pN stage and grade of HNC tumours. A negative correlation was observed between the age of the HNC patients versus pT stage, C stage and pN of the HNC tumour samples (Table IV).

Discussion

In this study, we evaluated possible associations between variants of the DNA repair gene *APEX1* and the risk of developing HNC. First, we identified potentially functional polymorphisms and genetic markers in the candidate gene by SSCP and sequencing the gene from a subset of 300 HNC patients and 300 control subjects. We identified three novel mutations: one in the 3′UTR region (13T < G) and two in the fourth exon (Ser129Arg, Val131Gly) of *APEX1*. Our study found that the homozygous and heterozygous variant genotypes of *APEX1* mutations, 13T < G, Ser129Arg and Val131Gly, were associated with a significantly increased risk of HNC among the smokers and non-smokers. Previous studies have identified five non-synonymous *APEX1* variants, which is rare for population-based studies (23,24). Many investigators have focused on the Asp148Glu variant, as it resides in the carboxy-terminus and no direct impact on endonuclease or DNA-binding activities was observed for Glu148Asp. However, variants outside the *APEX1* DNA repair domain, such as residues 61–318 or promoter regions, could be markers for the pathogenic SNP and may be informative (18). In our study, we found one variation at 13 residues (redox domain) and another two at 129 and 131 residues (DNA-repairing domain), which cover both the redox domain and DNA-repairing domain of *APEX1*. Structural prediction of two mutations (Ser129Arg, Val131Gly) in the coding region of *APEX1* has shown no major change in protein structure compared with wild-type protein. It is possible that in the present study mutations in both domains may have a combined effect on the repair capacity of APEX1. These variants may be associated with a reduced ability to communicate with

Table III. Adjusted OR of HNC associated with the mutations in final model stratified by smoking status

Mutations/genotype	Smokers				Non-smokers			
	Cases	Controls	[a]OR (95% CI)	*P-value	Cases	Controls	[a]OR (95% CI)	*P-value
13T < G								
TT	81	128	1.00 (ref)	0.0001	146	150	1.00 (ref)	0.007
GT	13	07	1.89 (0.75–4.82)		10	06	1.69 (0.61–4.71)	
GG	25	05	5.36 (2.02–14.20)		15	04	3.89 (1.28–11.88)	
Ser129Arg								
TT	67	133	1.00 (ref)	0.0001	146	156	1.00 (ref)	0.0001
GT	22	03	7.83 (2.32–26.47)		07	02	3.56 (0.73–17.28)	
GG	30	04	8.22 (2.86–23.64)		48	02	28.38 (6.83–117.93)	
Val131Gly								
TT	72	125	1.00 (ref)	0.0001	133	149	1.00 (ref)	0.07
GT	18	10	1.85 (0.84–4.08)		05	18	0.27 (0.09–0.72)	
GG	29	05	6.31 (2.41–16.54)		20	06	3.5 (1.39–8.84)	

[a]ORs were adjusted by age, sex and smoking status.
*P > 0.05, by chi-square test for trend.

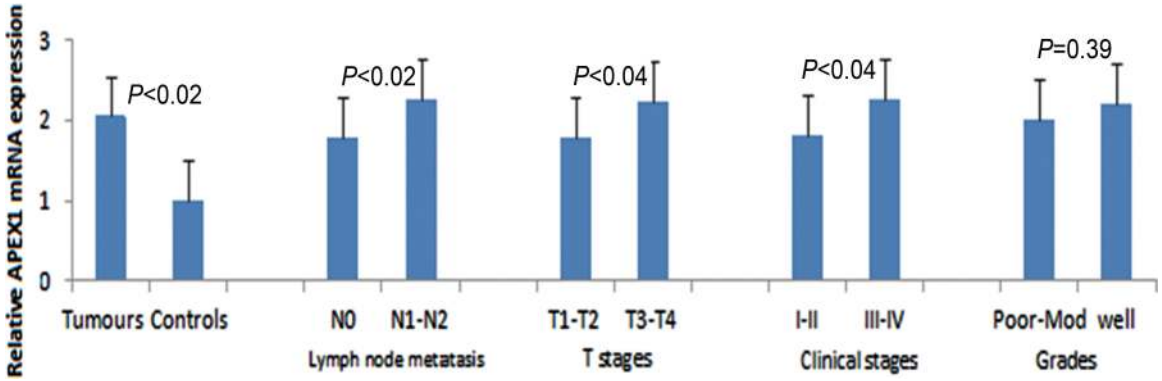

Fig. 3. mRNA expression of *APEX1* in HNSCC of study group 2. Columns plot comparing the *APEX1* mRNA levels of HNC and normal control samples, HNC samples with (N1–N2) and without lymph node metastasis (N0), with different pT stages, with clinical stage I–II and clinical stage III–IV and HNC samples with different grades. The *P*-values were computed using one-way analysis of variance.

other BER proteins, as even a slight change in DNA repair capacity may be detrimental and many functional studies are underpowered to detect subtle changes. Research has shown that APEX1 enhances the DNA-binding activity of NF-κB *in vitro* as well as NF-κB-dependent transcriptional activation *in vivo* (25,26). Deletion of the redox-sensitive domain of APEX1 has been shown to inhibit TNF-induced NF-κB activation and increased susceptibility to TNF-induced apoptosis (27,28).

In order to confirm our germ line results, that *APEX1* increases the risk of developing HNC in the Pakistani population, we planned another study to observe the expression level of this gene in the same population. As a result two important observations were made. First, deregulation of *APEX1* gene expression is a progressive event in the tumorigenesis of HNC. Our study observed that the *APEX1* level is increased in HNC when compared with normal tissue. Similar results have also been observed in osteosarcoma (29), pancreatic cancer (30), lung cancer (31), ovarian, gastro-oesophageal and pancreatico-biliary cancers (32). Second observation of our study is the positive correlation of *APEX1* level with pT stage, pC stage and positive lymph node metastasis. Earlier studies have demonstrated that different oxidative agents promote an increase of *APEX1* mRNA and protein expression (33,34). In case of lung cancer (35), ovarian (36), thyroid (37,38) and breast cancer (39), increased *APEX1* expression has been associated with higher aggressiveness of tumour (40). Accumulating evidence indicates that depletion of *APEX1* by the over-expression of antisense mRNA results in hypersensitivity to DNA-damaging agent hydrogen peroxide and methyl methanesulfonate in human cell lines (41) and oxidative stress induced *APEX1* over-expression in Chinese hamster cells (42). *APEX1*-compromised cells are more susceptible to oxidative stress mainly due to reduced redox and 3′phosphodiestrase activity impacting cell survival, pushing it towards apoptosis. This finding has therapeutic importance as increased *APEX1* levels in tumour cells have been shown to confer resistance to chemotherapeutic drugs perhaps via decrease in apoptosis (43).

Although this is not first report in relation to global context but Pakistani populations have not been analysed for such genetic changes and their association with different types of cancers. Nevertheless, our sample size (300 blood samples and 50 tumour samples) is relatively small. Further, validation studies with larger sample size and additional stratification to control potential confounding factors (ethnicity, smoking histories) are required to validate our findings.

In conclusion, our study demonstrates a significant association between *APEX1* mutations and HNC in Pakistani population at germ line level. Our study also confirms that the *APEX1* level is increased in HNC when compared with normal tissue. The *APEX1* level is further enhanced in more metastatic disease when compared with primary tumours. In line with previous findings, inhibition of DNA repair or redox or both activities of *APEX1* due to mutations or any inhibitors could potentially sensitise the tumour cells to therapeutic agents, making *APEX1* an attractive molecular target in the treatment of cancer.

Funding

This study was supported by grants from Higher Education Commission of Pakistan (HEC) grant number 2246.

Table IV. Correlations among clinical features and APEX1 gene expression of primary HNC[a]

Gender	Age	pT stage	C stage	pN stage	Grade	APEX1
Gender (*P*-value)	–0.194	0.017	0.124	0.038	–0.008	0.1
Age (*P*-value)	–	**–0.366** (<0.01)	**–0.362** (<0.01)	**–0.366** (<0.01)	–0.242	–0.204
pT stage (*P*-value)	–	–	**0.942** (<0.01)	**0.987** (<0.01)	**0.291** (<0.05)	**0.311** (<0.05)
C stage (*P*-value)	–	–	–	**0.929** (<0.01)	**0.289** (<0.05)	**0.287** (<0.05)
pN stage (*P*-value)	–	–	–	–	0.251	**0.304** (<0.05)
Grade (*P*-value)	–	–	–	–	–	0.142
APEX1 (*P*-value)	–	–	–	–	–	–

The expression levels of APEX1 for patient cohort 2 were based on the relative mRNA level. Bold values show statistically significant results.
[a]Spearman correlation coefficients.
$P < 0.05$. The *P*-values were computed using one-way analysis of variance.

Acknowledgements

All authors acknowledged the patients and healthy individuals who contributed to this research work. We also acknowledge hospital staff [Nuclear Oncology and Radiotherapy Institute Islamabad (NORI) and Military Hospital Rawalpindi (MH), Pakistan] for their cooperation.

Conflict of interest statement: The authors declared that they have no conflict of interest.

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