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TP53 Gene Alterations and P53 Protein Accumulation in Human Esophageal Cancer in Khartoum State, Sudan.

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ABSTRACT:
TP53 gene alterations were screened in 50 formalin fixed paraffin embedded (FFPE) human esophageal cancer (EC) specimens comprising 43 squamous cell carcinoma (SCC) and 7 adenocarcinoma (AC) using immunohistochemistry (IHC) and conventional PCR followed by DNA sequencing to detect p53 accumulation and mutation, respectively, in Khartoum state, Sudan.
TP53 gene mutations were detected in 20 (40%) of the 50 EC cases investigated. Five out of 13 (38.5%) of SCC cases that stained positive for p53 protein, showed TP53 mutations, only one (14.3%) of seven AC samples was positive for p53 protein without associated mutation and 4/7 (57.1%) showed TP53 mutations only. Immunohistochemical stain was positive in 14/50 (28%) of EC samples; 13 (92.9%) of them were SSC cases and one (7.1%) AC.
Five out of forty three (11.6%) of SSC and none of AC cases showed both immunohistochemical positive stain and mutation of TP53 gene while 8/43 (18.6%) of SSC and 1/7 (14.3%) of AC samples showed immunohistochemical positive stain but no TP53 gene mutation were present. Finally 15/50 (30%) of EC, 11 (73.3%) were SSC and 4 (26.7 %) AC showed immunohistochemical negative stain and positive TP53 gene mutations.
Immunohistochemical patterns of p53 were not related significantly to mutational analysis results in the cases examined. Mutation analysis is recommended to be done on the entire length of the gene using fresh tissue to complete the whole picture in future studies.
Key words: *TP53, immunohistochemistry, mutational analysis, esophageal cancer, Sudan.*

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I. Introduction

The incidence and mortality of ECare geographically varied with a high incidence in East Asia and Eastern/Southern Africa where SCC predominates over AC. EC is considered the eighth most common cancer worldwide and the sixth most common cause of cancer death with 400,000 case fatalities annually (1,2).

TP53 is one of the most commonly mutated genes in different types of human cancers and is altered in the early stages of lung, skin, head and neck, and esophageal cancers (3,4). Wildtype p53 protein has a short half-life and is found in small amounts in normal cells. Mutant p53 genes give rise to a mutated protein which can lead to loss of feedback inhibition or increase in the half-life of the mutated protein, which consequently accumulates inside cells, and can be detected using IHC. Theoretically, p53 overexpression, as an alternate marker for p53 gene mutations, is considered one of the immunohistochemical tests for cancer and precancerous changes. But in practice, the case is different because the correlation between IHC staining and gene alterations is not precise (5–7).

Billini *et.al.*2012 mentioned that the overexpression of p53 protein level usingIHC staining is used as an indicator of the mutant form of *TP53* gene, which has been shown to be a powerful marker of malignancy(8).

The purpose of this investigation was to characterize the spectrum of p53 alterations i.e. protein accumulation using IHC and gene mutational analysisusing polymerase chain reaction and Sanger sequencing in50 surgically resected EC samples and to evaluate associations with the available clinicopathological data.

II. Materials And Methods

Esophageal tissue specimens

Paraffin blocks of tissue specimens from 50 patients with EC were obtained from different hospitals in Khartoum State, Sudan, during the period 2013-2017. The patients were 20–93 years old (mean 56.5); 33 (66%) of them were females and 17 (34%) were males. All samples were previously diagnosed as SCC or AC.

Preparation of tissue sections.

Sections 4-5 μm thick were prepared from all tissue blocks. Part of these were adhered to ordinary slides and stained with Hematoxylin and eosin (H&E) for histopathology(9).Other sections were mounted on poly-Lysine-coated slides for immunostaining.

Immunohistochemistry

Sections from the FFPE specimens of EC, together with p53 positive and negative control slides were prepared for immunostaining.

Following deparaffinization in xylene, slides were rehydrated through a graded series of alcohol and placed in distilled water. Antigen retrieval was performed by boiling slides in antigen retrieval solution (1 mM citrate buffer, pH 6.0), ina water bath at 95º C for 30 min. After washing with PBS for 3 min endogenous peroxides activity were blocked with 3% hydrogen peroxide and methanol for 10 min. After washing with PBS for 3 min then slides were incubated with 100 μL of monoclonal mouse anti-humanantibody against p53 for 30 min at room temperature in a humidity chamber. Finally, the sections were washed in three changes of PBS, followed by adding 3, 3 di-amino benzidine tetrahydrochloride (DAB) as a chromogen for 5 min. After washing with distilled water for 3 min slides were counterstained with Mayer's hematoxylin for one min,then blued in running tap water for 7-10 minutes, then dehydrated, cleaned, and mount in DBX. Each tissue sectionwas evaluated independently by two investigators and discordant cases were discussed until consensus was reached.

Furthermore,a sample was defined to be positive for *TP53* when 10% of cells demonstrated staining (10).

Control sections used for determination of antibody reaction specificity included positive controls (sections of esophageal carcinoma immunoreactive for p53 protein and with p53 gene mutation detected by DNA sequencing) and negative controls (obtained by substituting the primary antibody with phosphate buffered saline). The tumors were classified according to the percentage of definite nuclear p53 protein immunoreactivity as follows:

1) Negative*P53* immunoreactivity: 0- 10% immunoreactive neoplastic cells;
2) Positive *P53* immunoreactivity: greater than 10% immunoreactive cells.

DNA extraction

DNA extraction was performed on FFPE tissue samples usingblack PREP FFPE DNA Kit from Analytikjena Company, Germany, according to manufacturer guides (11). Samples were then stored at 4 °C until used for PCR.

Polymerase chain reaction (PCR)

Exons 5 through 8 of the *TP53* gene were amplified using four pairs of specific oligonucleotide primers, as previously reported by Li et al. (12) and Makwane et al. (13) The sequences of these primers were as follows: Exon 5: sense 5'-TAC TCC CCT GCC CTC AAC AA-3', antisense 5'-CAT CGC TAT CTG AGC AGC GC-3'. Exon 6: sense 5'-TTG CTC TTA GGT CTG GCC CC-3', antisense 5'-CAG ACC TCA GGC GGC TCA TA-3'. Exon 7: sense 5'-TAG GTT GGC TCT GAC TGT ACC-3', antisense 5'-TGA CCT GGA GTC TTC CAG TGT-3'. Exon 8: sense 5'-AGT GGT AAT CTA CTG GGA CGG-3', antisense 5'-ACC TCG CTT AGT GCT CCC TG-3'. The size of PCR products was as follows: exon 5: 184 base pairs (bp); exon 6: 128 bp; exon 7: 117 bp, and exon 8: 141 bp. PCR reaction mixture (20 μL) was comprised of ready to use Intron Mastermix and 0.5 μL of each primer. A template, 1-3 uL of DNA was used. A Heal force DNA thermal cycler (Qingpu District, Shanghai, China) was utilized. After a hot start at 95 °C for 5 minutes 37 cycles of PCR were programmed as follows: 95°C for 45 sec, primer-specific annealing temperature for 45 sec in 59.7 ºC, 58.2 ºC, 59.3 ºC and 53.8 ºC for exon 5, 6, 7 and 8 respectively. 72°C for 45 sec. In all PCRs 5 minutes at 72 °C was added at the end of the last cycle followed by cooling step for an indefinitetime (∞)at 4 °C. The amplification products were visualized on ethidium bromide stained 2% gels.

Statistical analysis

Statistical analysis was performed with Statistical Package for the SocialSciences **(**SPSS) statistical software (IBM SPSS platform). Frequencies were calculated,correlation and chi-square were tested.

Ethical considerations

The study was approved by the institutional ethics committee, Sudan University of Science and Technology (No: DSR-IEC-13-05). Allpatients data used in this study have been anonymized to ensure patients privacy. Patients consent was difficult to obtain as most of them were dead or cannot be traced.

III. Results

Histopathology results:

The histopathological results of the EC biopsies revealed that 43/50 (86%) of the ECC specimens examined were SCC and the remaining seven (14%) were AC.

***TP53* gene mutations and protein accumulation**

Thirty-three patients out of 50 (66%) were female, and 17 (34%) were male, aged 20 to 93, with a mean age of 56.5 years.

TP53 mutations in exons 5-8 were detected in 20/50 (40%) of the EC specimens whereas p53 accumulation was observed in 14/50 (28%) of cases. Comparing DNA sequencing and IHC, there were 24/50 (48%) discordant cases, 9 of them were positive for P53 accumulation and negative for mutation detection, in addition to 15 were found to be negative for *TP53* accumulation with mutation detected. On the other hand, 21(42%) out of 50 specimens did not display *TP53* mutation or p53 accumulation. No associations were found between *TP53*gene alterations and clinicopathologic parameters, including patients' age and sex and tumor type. *P53* protein accumulation and *TP53* gene mutation were not related. SCC cases represents 43 (86%) and AC 7 (14%).

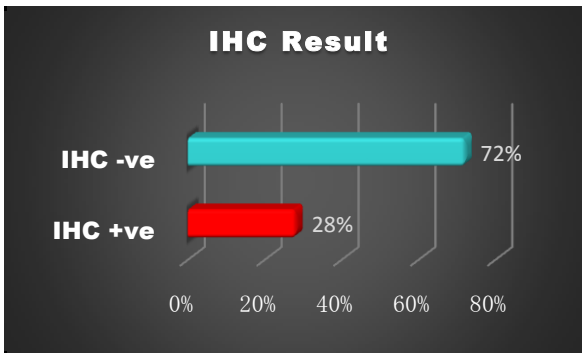

Fig. 1 Immunohistochemistry ratio of positive to negative cases.

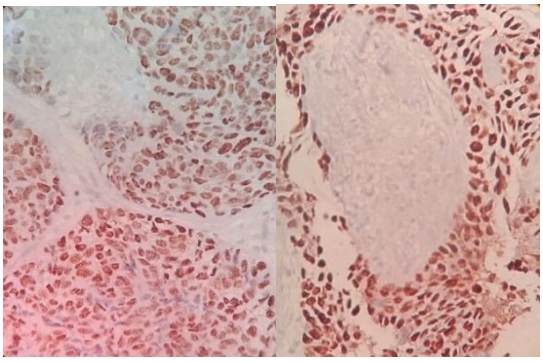

Fig. 2 IHC positive nuclear staining for P53.

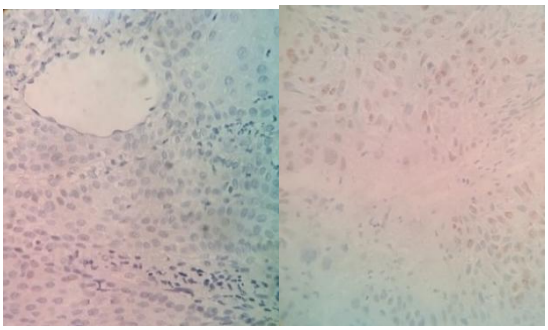

Fig. 2 IHC negative nuclear staining for P53.

Table 1. *TP53* Genotype and p53 Protein Accumulation in Esophageal Carcinoma

p53 mutation	No. of cases	p53 accumulation	
		positive	negative
None	30	9	21
Exon 5	17	5	12
Exon 6	1	0	1
Exon 5 & 6	1	0	1
Exon 7	0	0	0
Exon 8	1	0	1

Table 2.*TP53* Mutation and p53 Accumulation with Clinicopathologic Correlations

		TP53 mutation		IHC accumulation	
		Yes	No	Yes	No
Age group	>50	13	22	8	27
	<50	7	8	6	9
Gender	Male	8	9	3	14
	Female	12	21	11	22
Histology	SCC	16	27	13	30
	AC	4	3	1	6

Table 3. P53 protein accumulation and p53 gene mutation in esophageal cancer patients.

Histology	N	M	IHC (-ve)	IHC (+ve)
SCC	27	16	30	13
AC	3	4	6	1

N: normal, M: mutant.

Table 4. *TP53* immunostaining results compared to *TP53* gene mutational status in esophageal cancer patients.

Test results	Histopathology	patients
+ve IHC only	SCC	8
+ve Mutation only	SCC	11
+ve for both	SCC	5
-ve for both	SCC	19
+ve IHC only	AC	1
+ve Mutation only	AC	4
+ve for both	AC	0
-ve for both	AC	2

+ve = positive, -ve = negative

IV. Discussion

P53 protein in normal cells is a labile protein of low quantity. But cellular stress can generate an increase in the p53 protein level to compensate for its main function as a guardian of the genome (14,15).

Normally *TP53* levels are low; sometimes they may be even undetectable. However, the expression rate of *TP53* detected by immunohistochemistry has been reported to range from 33 to 70 % in esophageal cancer. *TP53* shows nuclear staining because of the accumulation of mutant *TP53*, which often has higher stability and is resistant to degradation, making it detectable by IHC. Accumulation of *TP53* detected by IHC does not necessarily imply gene mutation, but *TP53* over-expression in most of the cases (85 %) suggests an underlying mutation. So, p53 over-expression may be considered as an indicator of *TP53* gene mutation (16).

In this study *TP53* mutation in exons, 5 through 8 were detected in 40% of the EC patients whereas p53 accumulation was observed in 28% only. This result is comparable with the result of Uchino et al. 1996 who found a p53 mutation in 34% of esophageal cancer cases(17).

In a study done by Imazeki et al. 1992 mutations in the P53 gene were found in 80% of EC.This is a higher percentage compared to our finding which may be a false effect resulting from the small sample size (5 cases of EC) they used in their study (18).

The presentresultsindicate thatthe mutations in the *TP53* gene are not always associated with *P53* protein accumulation, as discordant cases reached 48%. Similarly,Coggi and his college in 1997 (19)detected P53 mutations in exons 5-8 in 53% of the ECs, p53 accumulation in 57% of cases;discordant cases represented 38%, whereas 27% did not display p53 mutation or accumulation.

No associations found between p53 alterations and clinicopathologic parameters, including patients age, sex, and tumor type and this seems to agree with the previous finding by Casson *et. al.* 1998 indicating that there were no statistical associations between different risk factors and the presence or absence of *TP53* alterations (20).

Generally, it is difficult to compare the results of various studies on PT53 gene mutations and p53 protein accumulation because of the differences of antibodies, antigen retrieval methods and solutions used and interpretation criteria followed to evaluate the results (21).

V. Conclusions

P53 abnormalities are very common in ECs especially exon 5 which comprises 19/21 (90.5%) of all mutations detected in exon 5-8. *TP53* gene mutation and p53 protein accumulation have a significant discordance, suggesting that p53 function may be inactivated by mechanisms other than mutation. So,*TP53* protein accumulation cannot be regarded as an indicator for genetic alteration in esophageal tumors.

Detection of p53 gene mutations is necessary in each case because gene therapy is one of the promising therapeutic modality in the near future.It has been suggested that mutations of the p53 gene are genetic events in the pathogenesis of esophageal SCCand gastric AC (16).

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CONFLICT OF INTEREST

Authors declares no conflict of interest.

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