Analysis of rs1048943 in the CYP1A1 gene in laryngeal cancer - a case control study [version 1; peer review: awaiting peer review]

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Abstract

Background: The CYP1A1 gene is essential in the metabolism of carcinogenic polycyclic aromatic hydrocarbons (PAHs). Numerous studies have investigated the links between the polymorphism Ile462Val rs1048943 A>G and its association with different cancers but the results have been variable. In this study, we studied rs1048943 in Sudanese patients diagnosed with laryngeal cancer.

Methods: This is a case-control study of 49 laryngeal cancer cases and 50 healthy controls. Restriction fragment length polymorphisms (RFLP) were used to analyse the fragment region of the CYP1A1 gene that contains rs1048943.

Results: The average age of the patients was 51.2 years old and the male to female ratio was 1:1. Well differentiated squamous cell carcinoma constituted most of the histopathological diagnoses. Ile462Val rs1048943 A>G was not found in any of the cases but was found in heterozygous form in four controls (p = 0.1331, OR 0.1044, 95% CI, 0.0054-1.9924).

Conclusions: Our study did not show a significant association of CYP1A1 Ile462Val rs1048943 A>G with laryngeal cancer. Future studies may need to test additional single nucleotide polymorphisms (SNPs) in laryngeal cancer tissue samples to identify the effect of those SNPs on laryngeal cancer.

Keywords

CYP1A1 gene, laryngeal cancer, rs1048943, squamous cell carcinoma.
Upper Aero-digestive tract cancers.
Introduction
Several epidemiological studies have suggested that tobacco smoking and alcohol consumption increase the risk of developing laryngeal cancer. Certain genetic polymorphisms have been identified as genetic risk factors specifically for laryngeal squamous cell carcinoma (SCC). Cytochrome P450 (CYP) enzymes are the key enzymes in phase I of the metabolism of carcinogenic polycyclic aromatic hydrocarbons (PAHs). PAHs are carcinogens associated with laryngeal cancer. Different studies have pointed to the involvement of CYP in procarcinogen activation. The CYP1A1 polymorphisms rs1048943 A>G and rs4646903 T>C have been the most frequently studied. CYP1A1 rs1048943 A>G is an amino acid change at codon 462. CYP1A1 rs4646903 T>C is characterized by the T-to-C mutation at nucleotide 3801 in the 3'-flanking region of the gene. Numerous studies have investigated the links between the polymorphisms rs1048943 and rs4646903 and the risk of laryngeal cancer, but the results appear to be inconsistent. The Ile462Val rs1048943 A>G polymorphism in the CYP1A1 gene is located in exon 7 in the heme binding region and leads to a doubling of enzyme activity. Heme plays a vital regulatory role in the cell.

The objective of this study was to find out if there was an association between the rs1048943 polymorphism and laryngeal cancer in Sudanese patients. The hypothesis was that the polymorphism might play a biological role in laryngeal cancer progression specifically in Sudanese patients.

Methods
Study design and participants
This was a case-control study carried out during the years 2019–2020. It involved 49 patients diagnosed with different grades and types of laryngeal cancers and 50 healthy controls. DNA samples for all participants were recruited from the upper aero digestive tract cancer (UADT) biobank. This biobank contains biological materials and data from cancer patients as well as control healthy individuals. These samples were collected from newly diagnosed cases to the biobank during the years 2014–2016 from the Radiation and Isotope Centre Khartoum (RICK), Sudan. The controls group was collected from healthy individuals recruited from the UADT biobank during the same period. The biobank includes a large number of DNA samples from both cancer patients and healthy controls; however, our selection was restricted to the patients diagnosed with cancer of the larynx only and controls were matched to patients by age and sex. The sample size was based on the availability of laryngeal samples in the biobank and matching controls. The biobank had a total number of 49 laryngeal cancer cases, so all were included.

Sample collection and DNA extraction
DNA samples from blood were obtained from the UADT biobank. The DNA had been previously extracted using the innuPREP mini Kit from Analytik Jena. DNA quantification (ng/μL) was determined with a 5 μl DNA sample using the NanoDrop 2000 (Cat. No. ND-2000, Thermo Fisher Scientific). The DNA samples were stored at -80°C.

Polymerase chain reaction method
The region that contains the changes of the nucleotides A>G which identifies rs1048943 was amplified by PCR using universal eubacterial primers to produce an amplicon of 226 bp. The primers were designed using Primer 3. Accordingly, the forward sequence was (CTCACCCCCCTGATAGGATAGTGCTAT) and the reverse sequence was (TTTGGAAATGCTCAGCAGCAGCAGCAGC). The reaction was performed in a total volume of 20 μl using the Maxime PCR premix kit i-startaq (Cat. No 25165): 0.3 μl of each primer, 14.7 μl DW and 5 μl of the DNA template. The PCR was performed in a Thermo cycler (Techne TC-412, UK) with an initial denaturation at 95°C for 2 minutes, followed by 35 denaturation cycles at 95°C for 30 seconds, primer annealing at 58°C for 30 seconds extension at 72°C for 30 seconds and a final extension at 72°C for 5 minutes. After that, gel electrophoresis was performed to detect the presence of the amplified DNA fragments in the PCR product. The gel contained agarose gel dissolved in a buffer to 1.5 % to which an ethidium bromide (0.5 μg/ml) was added. The size of the wild type band was 226 bp. The electrophoresis result was photographed with the Syngene InGenius LHR2 gel imaging system (Syngene, Woonsocket, RI, USA).

Restriction fragment length polymorphism (RFLP)
We used the NEBcutter V2.0 web tool provided by New England Bio labs to select the restriction enzyme specific to the position of the polymorphism. Accordingly, the 2-base pair product of the CYP1A1 gene was digested with the BsrD1 restriction enzyme. The enzyme recognized a short specific sequence of the nucleotide base, then catalysed hydrolysis (cleavage of the chemical bond that binds the nucleotide) by adding water molecules. The digestions were performed in a total volume of 31 μl. The reaction mixture consisted of 10 μl of the PCR product, 1 μl of the restriction enzyme and 2 ml of 10x buffer, with the volume adjusted by 18 μl sterile DW. Electrophoresis was performed on the digested product with 1.5 (w/v) agarose and 0.5 μg/ml ethidium bromide, and photographs were taken with the Syngene InGenius LHR2 gel documentation system (Syngene, Woonsocket, RI, USA). The sizes of the bands after digestion were 150 bp and 76 bp.

Data analysis
Statistical analysis was performed using Stata v11 software (Statacorp, College Station, Texas). Results were presented in frequencies for the grades and types of laryngeal cancer. Genotyping analysis was performed using the Chi-square test in order to estimate the association of the different alleles to laryngeal carcinomas. The statistical level of significance was set at p value 0.05.

Results
There were a total of 23 male and 26 female cases with a male to female ratio of 1:1. Their average age was 52.2 years old (range 13–80 years). The control group contained 23 males and 26 females with a male to female ratio of 1:1.7. Their average age was 51.6 (range 19–80) (Table 1).
Histopathological classification showed that 27 patients (55.1%) were diagnosed with well differentiated SCC, 12 (24.7%) with moderate SCC, 5 (10.2%) with poorly differentiated SCC and 1 (2%) with SCC without indication of the degree (Table 2). Other histopathological types (adenoid cystic carcinoma, diffuse large cell lymphoma, mucoepidermoid carcinoma, and laryngeal papillomatosis) had a sample of 1 (2%) participant each.

The genotypic distribution of rs1048943 A>G is shown in Table 3. The wild type homozygous genotype A/A was present in all the cases (100%) yet not the heterozygous (AG) or homozygous mutant (GG) genotype (0%). Of the controls, four had the heterozygous allele AG, 46 had the homozygous wild type AA genotype, and none had the GG genotype (p = 0.1331, OR 0.1044 and 95% CI 0.0054 to 1.9924) (Table 3).

**Discussion**

Laryngeal cancer is one of the most predominant cancers of the upper respiratory tract. It is rare in individuals under 60 years of age with three quarters of all diagnoses occurring in people over 60. In the current study, the mean age of the

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### Table 1. Sample characteristics.

|        | Males | Females | Male: Female ratio | Age range (years) | Average (years) |
|--------|-------|---------|--------------------|-------------------|-----------------|
| Cases  | 23    | 26      | 1:1                | 13-80             | 51.2            |
| Controls | 23  | 27      | 1:1.7              | 19-80             | 51.6            |

### Table 2. Histopathological diagnosis of the cases.

| Squamous cell carcinoma (SCC) | Number of patients (%) |
|-------------------------------|------------------------|
| Well differentiated SCC        | 25 (50%)               |
| Moderate differentiated SCC    | 12 (24%)               |
| Poorly differentiated SCC      | 5 (10%)                |
| Unknown differentiated SCC     | 5 (10%)                |

**Other types of laryngeal cancer**

- Adenoid cystic carcinoma: 1 (2%)
- Diffuse large cell lymphoma: 1 (2%)
- Mucoepidermoid carcinoma: 1 (2%)
- Laryngeal papillomatosis: 1 (2%)

### Table 3. The genotype distribution of rs1048943 in laryngeal and hypopharyngeal cancer patients and controls.

| Genotype | Cases % | Controls % | P value | OR (95% CI) |
|----------|---------|------------|---------|-------------|
| rs1048943|         |            |         |             |
| AA       | 50/50 (100) | 46/50 (92%) | 0.1331 | 0.1044 (0.0054 to 1.9924) |
| AG       | 0/50 (0)   | 4/50 (8%)  |         |             |
| GG       | 0/50 (0)   | 0/50 (0%)  |         |             |
participants was 51.2 years which is a rare age of onset for laryngeal cancer; however, young patients are expected to have a better prognosis.  

Worldwide, laryngeal cancer occurs more commonly in men than in women. In the Sudan cancer registry for the years 2009–2013, the ratio of men to women diagnosed with laryngeal was about 2.8:1. This contradicts the almost equal ratio we found in this study. This could be because all cases in this study were collected from Radiation and Isotope Centre of Khartoum (RICK). On the other hand, cancer registry cases were collected from numerous hospitals and pathology labs from all over Sudan.

In this study squamous cell carcinoma was the most common type of laryngeal cancer, which reflect the type of cells in the larynx. Well differentiated squamous cell carcinoma was the dominating grade, suggesting that the cancer was detected early.

The CYP enzymes, especially CYP1A1, play an important role in laryngeal cancers as well as in lung cancer, prostate cancer, colorectal cancer and renal cell carcinoma. Here, rs1048943 showed no association with laryngeal cancer (p = 0.1331). In view of the small sample size but with our high statistical power we deem these results to be reliable and accurate. We can conclude that rs1048943 might be a polymorphism found in different ethnicities but not directly linked to laryngeal cancer in Sudanese patients. Hence, it is necessary to study CYP1A1 using more advanced technology like next generation sequencing (NGS) to identify SNPs associated with laryngeal cancer.

**Conclusion**

To sum up, the present study did not find an association between rs1048943 and laryngeal cancer in Sudanese patients.

**Data availability**

DRYAD: rs1048943 in CYP1A1 gene in laryngeal cancer-Sudanese study. https://doi.org/10.5061/dryad.j3tx95xcm.

This project contains the following underlying data:

- Sudan-larynx-SNP_analysis.xlsx (demographics and clinical information of participants).

Data are available under the terms of the Creative Commons Zero “No rights reserved” data waiver (CC0 1.0 Public domain dedication).

**Consent for publication**

Written informed consent for publication of the participants’ details was obtained from the participants.

**Ethical consideration**

The project was approved by the National Research Ethical Review Committee Health Research Council, Republic of Sudan National Ministry of Health in 2014 (NO:UADT/6.2014). All samples are part of the UADT cancer biobank. A signed consent form was submitted by all study participants.

**Authors contributions**

Marwa Abdalwahab conducted the laboratory work and contributed in Manuscript writing. Dr. Mona Ellaiithi was the principle investigator and writer of the manuscript.

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