Sudan University of Sciences and Technology

College of Graduate Studies

# Histopathology, Immunohistochemistry and Molecular Study of *Tumor Protein 53* Gene Mutations in Esophageal Cancer among Sudanese Patients

**دراسة التشخيص النسيجي المرضي و الكيمياء النسيجية المناعية والفحص الجزيئي**

**لطفرات جين البروتين الورمي 53 في سرطان المرئ لدى المرضى السودانيين**

A Thesis Submitted for Fulfillment of the Requirement of the PhD. in medical laboratory sciences

**Submitted by**:

Rihab Elmahdi Elfaki.

M.Sc medical laboratory sciences – SUST (2011).

**Supervised by**:

Prof. Ahmed abdul Rahim Gameel.

**Co. supervisors:**

Dr. Mohammed Siddig Abdelaziz.

Dr. Munsour M. Munsour.

*2018*

## الآية

بسم الله الرحمن الرحيم

قال تعالى:

# «قَالُوا سُبْحَانَكَ لَا عِلْمَ لَنَا إِلَّا مَا عَلَّمْتَنَا ۖ إِنَّكَ أَنتَ الْعَلِيمُ الْحَكِيمُ» ﴿32﴾

سورة البقرة

# DEDICATION

To my parents Mahdi & Abda

To my Pro. & sis. Mohamed, Fatima, Marwa and Amr

To the love of my lifetime Omer

And my lovely kids Mohamed & Reem

To all beautiful people in my life

Specially Sana, tyseer and Randa

Those who step by and supporting me all the way

And till the end

To allCompanions of suffering, the fibromyalgia

patients around the world

With big love

# ACKNOWLEDGEMENT

First of all I thank God for making all my dreams true and giving me the grace of ambition and the power to seek the knowledge whenever possible, and filling my life with exceptional peoples, those lighting the dark nights of my life. Special thanks to everyone who participated in this work by advising, exchanging ideas or constructive criticism to make this work possible.

I am extremely grateful to prof. Ahmed Abdel Rahim Gameel, Pathology Department, Faculty of Veterinary Medicine, University of Khartoum, for his patience, tolerating me all the way and being a great example for a person who dedicated himself to knowledge with humbleness of scientists.

I owe my most grateful thanks to Dr. Mohammed Siddig Abdulaziz, vice rector of Sudan University of Science and Technology, Department of Histopathology and Cytology, for his invaluable support, encouragement, guidance, and patience while working on this study.

I am grateful to Dr. Hisham Noureldayem Eltyeb, Department of Microbiology, College of Medical Laboratory Sciences, Sudan University of Science and Technology for the opportunity to work with him, and I appreciate his guidance, and unlimited support to ensure the completion of the research in the best way.

I appreciate the role of Dr. Munsour M. Munsour for his patience, guidance, help and collaboration. Also special thanks to all members of research laboratory in Sudan University, specially Sohair Ramadan and Umsalama Baballah.

I would like also to thank Prof. Ahmed Abd Elbadiee and Sara Saad from El-Alem Laboratory, Dr. Saifeldin Mohammed Elzain and Samah Taj Elsir from national laboratory of health, Dr. Azaa Zulfu, Ayat Salah, Alaa Mustafa and Mahmood Ashry from Khartoum hospital, Abeer Musa from Soba hospital, Mohanad Mostafa, Nihal Babiker Yaseen and Eman Ahmed from Alrahma Laboratory, Montaser Elsaiegh, Ahmed Jalal and Israa Abdulrahman Dhary from Total Lab Care Laboratory, Majdy Mostafa from Alnelain Center, Nemat Ismaeel and Lubna Mohammed from Bahry hospital, Dr. Khalid Mohsen, Dr. Rabab Naser, Taqwa Basheer, Nihal Babiker and Samawal Elimam from Umdurman Teaching Hospital, Nahla Gaafar, Rania Ahmed, from Ibn Sina Hospital, Dr. Elsadiq Ahmed Adam and Baqeea Hassan Abusenena from Alribat National Hospital, Dr. Mahjoob Tajeldeen Othman and Naglaa Mohammed from Military Hospital for their great help in sample collection.

I am indebted to Nahla Hashim and Rana Elnoor Ibraheem for their valuable advices and help in immunohistochemistry staining. Finally, I would like to thank my family and friends, particularly my son Mohamed and my daughter Reem, for their patience and unending help in the house chores.

I appreciate the role of Statistic and Archives Department staff in Omdurman Teaching Hospital, Radioisotope Center Khartoum and National Health Laboratory, Finally, my sincere thanks to those whose names have fallen inadvertently.

# ABSTRACT

The protein product of the normal tumor protein 53 (TP53) gene performs an essential function in cell cycle control and tumor suppression, and the mutation of a TP53 gene is an essential step in the development of many cancers. Despite the reported association of TP53 gene mutations with many human cancers, the comprehensive computational analysis of single nucleotide polymorphisms (SNPs) is of paramount importance in predicting functional and structural impacts of specific SNPs. The aim of this study was to detect TP53 gene mutations in exon 5-8 in Sudanese patients with esophageal cancer (EC). In this study 204 esophageal cancer specimens were collected from different hospitals in Khartoum state, and investigated using hematoxylin and eosin (H&E) stain for histopathologic classification of EC, in addition to immunohistochemistry (IHC) stain to detect p53 accumulation, *TP53* gene alterations were investigated using Deoxyribonucleic acid (DNA) extraction from the same specimens followed by polymerase chain reaction (PCR) and finally DNA sequencing were screened in 50 formalin fixed paraffin embedded (FFPE) specimen due to DNA scarcity or absence in part of the specimens and degradation of the DNA in the rest of them. Computational analysis was performed using different algorithms to screen for deleterious single nucleotide polymorphisms (SNPs). In the 204 specimens used, their patients age ranged from 18-93 years, mean 60 years; those who were <60 years of age were classified as the young group which comprises 72/204 (35.3%) and classified the other as the elderly group 132/204 (64.7%). Males were 76 (37.3%) and females were 128 (62.7%). Histopathologically, the EC specimens were classified as squamous cell carcinoma (SCC; n= 170),

Adenocarcinoma (AC; n= 21) and undifferentiated carcinoma (n= 13). IHC positive samples were 56 (27.5%) and negative samples were 148 (72.5%), the study found there is a significant relationship between sex and histopathologic diagnosis with SCC being more prevalent in females, AC and UDC in males.

The 50 EC specimens used for detection of TP53 gene alterations comprised 43 SCC and 7 AC. The results also demonstrated that there are synonymous SNPs (sSNPs) (n= 4) and non-synonymous SNPs (nsSNPs) (n= 6) in the TP53 gene that may be deleterious to p53 structure and function. The 50 specimens selected for sequencing, their age range from 20-93 years, with (<60 years) group comprising 20/50 (40%) and (>60 years) group was 30/50 (60%), also 33(66%) of them were female and 17 (34%) were males and 43 (86%) Were diagnosed as squamous cell carcinoma (SCC) and 7 (14%) as adenocarcinoma (AC), Additionally, TP53 gene alterations were found in 20/50 (40%) of samples. Six out of ten of TP53 gene alterations occurred in exon 5, two in each of exons 6 and 8. Only one SNP in position E298Q was predicted to have a neutral effect and other SNPs were predicted to be disease related according to MutationTaster software. A total of 15/40 (37.5%) of SCC samples were found to be altered, 12/15 (80%) of them exist in exon 5 and 1/15 (6.7%) in exon 6, whereas AC showed a higher rate of alterations 4/7 (57.1%) with 100% involvement of exon 5, and no involvement of exon 6 and 8. *TP53* gene alterations 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* alterations, only one (14.3%) of seven AC samples was positive for p53 protein without associated genetic alterations and 4/7 (57.1%) showed *TP53* mutations only.

Immunohistochemical stain For P53 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 alterations 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 mutations were present. Finally 15/50 (30%) of EC; 11 (73.3%) SSC and 4 (26.7 %) AC, showed immunohistochemical negative stain and positive TP53 gene alterations. Statistically there is no correlation or significant association between TP53 gene alteration and sex, age of patients or tumor type. The study concluded that alterations of exon 5 in TP53 gene of EC patients were the most frequent. Genomic results have identified a higher TP53 mutation rate in esophageal AC in contrast to SCC. Also Immunohistochemical patterns of p53 were not significantly related 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.

# المستخلص

المنتج البروتيني للجين TP53 الطبيعي له وظيفة أساسية في التحكم في دورة الخلية وتثبيط الورم ، ويعتبر حدوث طفرة في جين TP53 خطوة أساسية في تطور العديد من أنواع السرطان .على الرغم من وجود ارتباط بين طفرات الجين TP53 مع العديد من أنواع السرطان البشري ، إلا أن تحليل المعلوماتية الحيوية الشامل الخاص بتعدد الأشكال النيوكليوتيدية المفردة (SNPs) ذو أهمية كبيرة لتوقع التأثيرات الوظيفية و التركيبية لتعدد الأشكال النيوكليوتيدية المفردة المحددة. الهدف من هذه الدراسه هو الكشف عن طفرات جين في اكزون 5-8 في مرضي سرطان المرئ السودانيين. في هذه الدراسة جمعت 207 عينة سرطان مرئ, تم اجراء فحص باستخدام صبغة الهيماتوكسيلين و الايوسين (H&E) علي جميع العينات للتصنيف التشخيصي النسيجي لسرطان المرئ, بالاضافة الي صبغة الكيمياء النسيجية المناعية (IHC) لفحص تجمع بروتين 53 أما تغيرات جين TP53 فقد تم فحصها عن طريق استخلاص الحمض النووي الريبوزي منزوع الأكسجين من نفس العينات يليه تفاعل البوليميراز المتسلسل التقليدي (PCR) ومن ثم تم اختبار تسلسل الحمض النووي علي 50 عينة من العينات المثبتة في الفورمالين و المطمورة في البارافين (FFPE) لسرطان المريء البشري (EC) نسبة لقلة أو عدم ظهور حمض نووي في بعض العينات, و تكسر للحمض النووي في الأخري. من ثم تم إجراء التحليل الحسابي باستخدام خوارزميات مختلفة للتحري عن ال SNPs الضارة. في المائة و أربع عينات المستخدمة أعمار المرضي تتراوح بين 18-93 سنة, متوسط عمر المرضي 60 عاما, لقد تم تصنيف من هم أقل من 60 عاما كمجموعة شابة و التي تشكل 72\204 (35.3%) و صنفت المجموعة الأخري كمجموعة كبار السن 132\204 (64.7%). الرجال شكلوا 76 (37.3%) و النساء 128 (62.7%). نسيجيا تم تقسيم عينات سرطان المرئ الي سرطان الخلايا الحرشفية (عدده= 170) و السرطان الغدي (عدده= 21) و السرطان غير المتمايز (عدده= 13) العينات الموجبة لصبغة كيمياء النسيج المناعية كانت 56 (27.5%) و العينات السالبة 148 (72.5%). الخمسين عينة التي استخدمت للكشف عن تغيرات جين TP53 ثلاثة و اربعون منها كانت عبارة عن سرطان الخلايا الحرشفية و سبعة من السرطان الغدي. تثبت النتائج أن هناك SNPs مترادفة (sSNPs) (عدده= 4) و SNPs غير مترادفة (nsSNPs) (عدده= 6) في الجين TP53 التي قد تكون ضارًة لتركيبه و وظيفته. كشفت الدراسة عن وجود علاقة قوية بين الجنس و

التشخيص النسيجي حيث لوحظ انتشار سرطان الخلايا الحرشفية اكثر وسط النساء و السرطان الغدي وغير المتمايز بين الرجال. الخمسون عينة المختارة لفحص تسلسل الحمض النووي, تراوحت اعمار المرضي بين 20-93 سنة, المجموعة العمرية (أقل من 60 سنة) شكلت أغلبية بنسبة 50\20 (40%) و المجموعة العمرية (أقل من 60 سنة) شكلت 50\30 (60%), أيضا 33 (66%) منها كانت سيدات و 17 (34%) رجال، و43 (86%) منها شخصت كسرطان الخلايا الحرشفية (SCC) و7 (14%) كسرطان غدي (AC). بالإضافة إلى ذلك ، تم العثور على تغيرات في جين TP53 في 50\20 (40%) من العينات .حدثت ستة من أصل عشرة من هذه التغيرات في جين TP53 في exon 5 ، وكان هناك تغيران في exon 6 وحدثت تغيرين آخرين في exon 8. وتوقع أن يكون SNP واحدة فقط في الموضع E298Q لها تأثير محايد وأن من المتوقع أن تكون ال SNPs الأخرى ذات علاقة بالأمراض وفقا لبرنامج MutationTaster. تم العثور على ما مجموعه 40\15 (37.5%) من عينات سرطان الخلايا الحرشفية (SCC) التي تحتوي تغيرات، 15\12 (80%) منها موجودة في exon 5 و 15\1 (6.7%) فيexon 6 ، في حين حققت (AC) معدل أعلى من التغيرات 7\4 (57.1%) مع نسبة 100٪ اصابة ل exon 5 و لم تسجل أي اصابات ل exon 6 و exon 8 .تم الكشف عن تغيرات الجين TP53 في 20 (40 ٪) من 50 من حالات سرطان المرئ التي تم التحقيق فيها .أظهر خمسة من أصل 13 (38.5٪) من حالات SCCالمصبوغة ببروتين p53 وجود تغيرات في جين TP53 ، عينة واحدة فقط (14.3٪) من أصل سبع عينات سرطان غدي كانت موجبة لبروتين p53 بدون تغيير جيني مصاحب و 7/4 (57.1٪) أظهرت تغيرات جينية فقط .كانت صبغة الكيمياء النسيجية المناعية لبروتين 53 إيجابية في 50/14 (28 ٪) من عينات سرطان المرئ. 13 (92.9 ٪) منهم من حالات SSC وواحد (7.1 ٪) AC . أظهر خمسة من أصل ثلاثة وأربعين (11.6 ٪) من SSC و لم تظهر أي من حالات AC نتيجة ايجابية لصبغة الكيمياء النسيجية المناعية أو تغير الجين TP53 في حين 43/8 (18.6 ٪) من SSC و 7/1 (14.3 ٪) من عينات AC أظهرت نتائج إيجابية لصبغة الكيمياء النسيجية المناعية مع وجود تغيير جيني . وأخيرا 50/15(30٪) من سرطان المرئ ، 11(73.3%) كانت SSC و 4 (26.7٪) AC أظهرت صبغة كيميائية نسيجية مناعية سلبية مع تغيير جيني. احصائيا لم يوجد اي ارتباط او فرق ذو دلالة احصائية بين تغيرات جين TP53 و الجنس, عمر المرضي او نوع الورم. خلصت الدراسة الي أن التغيير في جين TP53 في exon 5 في مرضى سرطان المريء هو الأكثر شيوعا .وقد حددت النتائج الجينية معدل تغييرات أعلى في AC المريء

على النقيض من .SCC كذلك لم تكن الأنماط الكيميائية المناعية للـ p53 مرتبطة بشكل كبير بنتائج التحليل الجيني في الحالات التي تم فحصها .يوصى بتحليل التغيرات الجينية على طول الجين باستخدام الأنسجة غير المعالجة كيميائيا لإكمال الصورة الكاملة في الدراسات المستقبلية.

# List of contents


| No. | Subject | Page |
| --- | --- | --- |
| | A verse from Holy Quran | I |
| | Dedication. | II |
| | Acknowledgement. | III |
| | Abstract (English). | V |
| | Abstract (Arabic). | VIII |
| | List of contents. | XI |
| | List of figures. | XIII |
| | List of tables. | XV |
| | List of abbreviations. | XVII |
| | Chapter 1 Introduction. | |
| 1.1 | Introduction. | 1 |
| 1.2 | Rationale. | 5 |
| 1.3 | Objectives. | 5 |
| 1.3.1 | General objectives. | 5 |
| 1.3.2 | Specific objectives. | 5 |
| | Chapter 2 Review of literature. | |
| 2.1 | Anatomy and histology of the esophagus. | 6 |
| 2.2 | Physiology of the esophagus. | 7 |
| 2.3 | Pathology of the esophagus. | 8 |
| 2.4 | Epidemiology of esophageal cancer. | 11 |
| 2.5 | Preventive factors | 14 |
| 2.6 | Etiology of esophageal cancer. | 15 |
| 2.7 | Signs and symptoms of esophageal cancer. | 18 |
| 2.8 | Diagnosis of esophageal cancer. | 19 |
| 2.9 | Treatment | 23 |

| | | |
|---|---|---|
| 2.10 | Complication of esophageal cancer. | 29 |
| 2.11 | Bioinformatics tools used in mutational analysis. | 31 |
| | Chapter 3 Material and methods. | |
| 3.1 | Type of the study | 35 |
| 3.2 | Study area. | 35 |
| 3.3 | Study samples. | 35 |
| 3.4 | Samples (materials). | 35 |
| 3. 5 | Sample processing. | 36 |
| 3.6 | Immunohistochemistry protocol. | 36 |
| 3. 7 | Molecular genetics | 38 |
| 3.8 | Tools and web servers used in bioinformatics analysis of DNA sequences. | 40 |
| 3.9 | Statistical analysis, | 41 |
| 3.10 | Ethical considerations. | 41 |
| | Chapter 4 Results. | |
| 4.1 | H&E results. | 42 |
| 4.2 | Immunohistochemical staining results. | 43 |
| 4.3 | Mutational analysis result. | 46 |
| 4.4 | Statistical analysis. | 59 |
| | Chapter 5 Discussion, conclusion and recommendations. | |
| 5.1 | Discussion. | 66 |
| 5.2 | Conclusion. | 72 |
| 5.3 | Recommendations. | 73 |
| | References. | 74 |
| | Appendices. | 91 |

# List of figures


| figure no. | Figures | Page |
|---|---|---|
| 1 | Percentage of male and female of EC patients. | 42 |
| 2 | a&b IHC positive nuclear staining for P53. | 43 |
| 3 | a&b IHC negative nuclear staining for P53. | 44 |
| 4 | Age groups frequency. | 45 |
| 5 | Distribution of different mutation types. | 47 |
| 6 | Distribution of TP53 coding synonymous SNPs (sSNPs), coding nonsynonymous SNPs (nsSNPs) and INDEL through exon 5-8 in P53 gene. | 47 |
| 7 | Distribution of deleterious and benign nsSNPs by SIFT, PolyPhen, and I-Mutant Suite | 48 |
| 8 | Mutation of alanine into aspartic acid at position 161. | 56 |
| 9 | Mutation of a serine into asparagine at position 215. | 56 |
| 10 | Mutation of arginine into proline at position 175. | 57 |
| 11 | Mutation of lysine into glutamic acid at position 164. | 57 |
| 12 | Mutation of a methionine into a valine at position 160. | 58 |
| 13 | Mutation of glutamic acid into glutamine at position 298. | 58 |
| 14 | Number of Affected Exons frequency. | 61 |
| 15 | Affected Exons frequency. | 61 |
| 16 | Invasive SCC. H&E x 100. | 101 |
| 17,18 | Dysplasia of basal cells with Subepithelial neoplastic cells. H&E x 40. | 101 |
| 19,20 | Downward projections of basal cells. H&E x 100. | 102 |
| 21 | Trabecular and cord-like Pattern of neoplastic cells. H&E x40. | 102 |
| 22 | Trabecular and cord-like Pattern of neoplastic cells. H&E x400. | 102 |
| 23 | Papillary-like Pattern of neoplastic cells. H&E x40. | 103 |
| 24 | Cord-like pattern of neoplastic cells. H&E x 40. | 103 |
| 25 | Packed neoplastic cells separated by thin strands. H&E x 400. | 103 |
| 26,27 | Intact squamous epithelium with basal dysplasia. H&E x 40. | 104 |
| 28 | SCC Epithelial keratinization H&E x100. | 104 |
| 29 | SCC Multiple epithelial pearls. H&E x100. | 104 |
| 30 | SCC keratinocytes with intercellular bridges. H&E x 100. | 105 |
| 31 | SCC Intercellular bridges H&E x 1000. | 105 |
| 32 | SCC vacuolated cells with pyknotic nuclei. H&E x 100. | 105 |
| 33 | Enlaged vacuolated cells, Pagetoid appearance. H&E x 40. | 106 |
| 34 | Enlarged cells, pagetoid appearance. H&E x 40. | 106 |
| 35 | SCC Groups of vacuolated cells. H&Ex100. | 106 |

| | | |
|---|---|---|
| 36 | SCC. Necrotic tumor tissue H&E x 100. | 107 |
| 37 | SCC. Necrotic tumor tissue with globules. H&E x 100. | 107 |
| 38 | Higher magnification of H&E x 400. | 107 |
| 39 | SCC Suspected bacterial Colonies. H&E x 400. | 107 |
| 40 | Small cell carcinoma H&E x 40. | 108 |
| 41 | Small cell carcinoma, cells resemble lymphocytes. H&E x 100. | 108 |
| 42 | PDSCC. Spindle shaped cells HE x 100**.** | 108 |
| 43 | PDSCC with clear cells HE x 400. | 108 |
| 44 | SCC Tendancy to form Glandular structures. H&E x 100. | 109 |
| 45 | SCC Cellular pleomorphism H&E x 400. | 109 |
| 46 | PDSCC. H&E x 400. | 109 |
| 47 | SCC Cells nests Surrounded by scnty stroma. H&E x 100. | 109 |
| 48 | SCC Cells nests Surrounded by wide stroma. H&E x 100. | 110 |
| 49 | Pseudosarcomatous SCC H&E x 40. | 110 |
| 50 | Adenocarcinoma H&E x40. | 110 |
| 51 | Adenocarcinoma H&E x100. | 110 |
| 52,53 | Cystic AC H&E x 100. | 111 |
| 54 | AC Resembling gastric Mucoa. H&E x 40. | 111 |
| 55 | Higher magnification of Fig. 39. H&E x 100. | 111 |
| 56,57 | Both figures from SCC section. Tissue fraction lined by respiratory-like pseudostratified columnar epithelium. HE x 100. | 112 |
| 58 | Sub-epithelial glands HE x 40. | 112 |
| 59 | Sub-epithelial glands. | 112 |
| 60 | Neutrophils in tumor tissue and blood vessel. HE x 100. | 113 |
| 61 | Tumor cells in a pool of blood. H&E x 40. | 113 |
| 62 | Higher magnification of Fig, 49.HE x 400. | 113 |

# List of tables


| Table No. | Tables | Page |
|---|---|---|
| 1 | *TP53* gene primers set. | 40 |
| 2 | Distribution of EC different histopathologic subtypes. | 43 |
| 3 | *TP53* Genotype and p53 Protein Accumulation in Esophageal Carcinoma | 44 |
| 4 | *TP53* Mutation and p53 Accumulation with Clinicopathologic Correlations. | 45 |
| 5 | P53 protein accumulation and p53 gene mutation in esophageal cancer patients. | 45 |
| 6 | The Prediction Results of nsSNPs of *TP53* Using SIFT, PolyPhen and I-Mutant 3.0 algorithms. | 48 |
| 7 | List of variants that were analyzed using SIFT algorithm. | 48 |
| 8 | Prediction of functionally significant nsSNPs by PolyPhen 2.0 algorithm. | 49 |
| 9 | Prediction of nsSNPs stability status by I Mutant 2.0 algorithm. | 49 |
| 10 | SNPs information and frequency among different samples. | 49 |
| 11 | Prediction of functionally significant nsSNPs, by PhD-SNP. | 50 |
| 12 | Summary of SNP Characteristics identified in *TP53*gene exon 5, 6 and 8. | 50 |
| 13 | FATHMM server; cancer association predictions result of the non-synonymous changes found in *TP53* gene exon 5-8. | 50 |
| 14 | Age group, sex, IHC result and histopathologic diagnosis frequencies of 204 samples. | 59 |
| 15 | Relationship between immunohistochemistry result and histopathologic diagnosis. | 59 |
| 16 | Relationship between age group and histopathologic diagnosis. | 59 |
| 17 | Relationship between sex and histopathologic diagnosis. | 60 |
| 18 | Age group, sex, mutational status, IHC result and histopathologic diagnosis frequencies (50 sample). | 60 |
| 19 | Relationship between histopathologic diagnosis and mutational status. | 62 |
| 20 | Relationship between histopathologic diagnosis and number of affected exons. | 62 |
| 21 | Relationship between histopathologic diagnosis and number of affected exons. | 63 |

22 Relationship between histopathologic diagnosis and immunohistochemistry result. 63

23 Relationship between immunohistochemistry result and mutational status. 64

24 Relationship between immunohistochemistry result and number of affected exons. 64

25 Relationship between immunohistochemistry result and affected exon. 65

# List of abbreviations

| | |
|---|---|
| AA | Amino Acids. |
| AC | Adenocarcinoma. |
| BE | Barrett’s Esophagus. |
| BLAST | Basic Local Alignment Search Tool. |
| BMI | Body Mass Index. |
| CCAR2 | Cell Cycle and Apoptosis Regulator 2. |
| CI | Confidence Interval. |
| CIS | Carcinoma In Situ. |
| CRP | C-Reactive Protein. |
| CT | Computed Tomography. |
| dbSNP | Database of Single Nucleotide Polymorphism. |
| DNA | Deoxyribonucleic Acid. |
| DPX | Distyrene Plasticizer Xylene. |
| EAC | Esophageal Adenocarcinoma. |
| EC | Esophageal Cancer. |
| EGFR | Epidermal Growth Factor Receptor. |
| ESD | Endoscopic Submucosal Dissection. |
| ESSC | Esophageal Squamous Cell Carcinoma. |
| FASTA | FAST Algorithm. |
| FFPE | Formalin Fixed Paraffin Embedded Tissue. |
| GERD | Gastroesophageal Reflux Disease. |
| GI | Gastrointestinal. |

| | |
|---|---|
| GWAS | Genome-Wide Association Study. |
| H&E | Hematoxylin and Eosin. |
| HGIN | High Grade Intraepithelial Neoplasia. |
| HOPE | Have yOur Protein Explained. |
| IHC | Immunohistochemistry. |
| IL-6 | Interleukin 6. |
| JEOG | The Japanese Esophageal Oncology Group. |
| MRI | Magnetic Resonance Imaging. |
| MT | Mutant Type. |
| nsSNP | Non Synonymous Single Nucleotide Polymorphism. |
| NCBI | National Center for Biotechnology Information. |
| NSAIDs | Non-Steroidal Anti-Inflammatory Drugs. |
| OS | Overall Survival. |
| P53 | Protein 53. |
| PCR | Polymerase Chain Reaction. |
| PDF | Portable Document Format. |
| PhD-SNP | Predictor of human Deleterious Single Nucleotide Polymorphisms. |
| PKM2 | Pyruvate Kinase M2. |
| p-mTOR | Phosphorylated Mammalian Target Of Rapamycin. |
| PolyPhen | Phenotype Polymorphism. |
| PPI | Proton Pump Inhibitors. |
| RCT | RadioChemoTherapy. |
| RPM | Revolutions per Minute. |
| RR | Relative Risk. |

| | |
|---|---|
| SCC | Squamous Cell Carcinoma. |
| SIFT | Sorting Intolerant From Tolerant. |
| SNP | Single Nucleotide Polymorphism. |
| sSNP | Synonymous Single Nucleotide Polymorphism. |
| SVM | Support Vector Machine. |
| TNF | Tumor Necrosis Factor. |
| TP53 | Tumor Protein 53. |
| VEGF | Vascular Endothelial Growth Factor. |
| WT | Wild Type. |

# CHAPTER ONE

## INTRODUCTION

# CHAPTER ONE
# INTRODUCTION

## 1.1. Introduction

Esophageal cancer (EC) is the eighth most common cancer and the sixth most common cause of cancer death worldwide. The vast majority of cases occur in underdeveloped regions, and with a higher frequency in men compared to women (Wang *et al.* 2010; Kim & Shah, 2017).

A high-risk area for EC is known as the so-called (Asian esophageal cancer belt), which stretches from north central China into northern Iran, where esophageal squamous cell carcinoma predominates (Taghavi *et al.* 2010).

Histologically EC can be classified into two main types: esophageal squamous cell carcinoma (SCC) and esophageal adenocarcinoma (AC). These two cancer types differ not only histologically, but also with respect to their incidence trends, populations they affect, and risk factors (Kamangar *et al.* 2009).

The main risk factors for AC are gastroesophageal reflux and obesity and those for SCC are tobacco smoking and heavy alcohol drinking (Ljung *et al.* 2013).

Traditionally EC is considered one of the fatal cancers that has not been studied attentively all around the world, taking in account its aggressiveness and reduced survival rate; this is because it is largely asymptomatic in its early stages. But the situation is improving with better diagnostic techniques that allow for earlier detection and treatment. The overall 5-year survival

rate for EC ranges between 10% and 15%; it ranks sixth among all cancers in mortality rate ( Corwin, 2008; Wang *et al.* 2012; Zhang, 2013).

About 50% of cancers extend beyond the primary local region at the time of diagnosis and almost 75% of surgically treated patients have proximal node metastasis (Wang *et al.* 2010).

The *TP53* tumor suppressor gene has an open reading frame of 393 amino acids long, located at the short arm of human 17 chromosome. This protein plays an important role in the cellular integrity. Loss of *P53* activity predisposes cells to the acquisition of oncogenic mutations and may favor genetic instability (Lehrbach *et al*., 2003; Cao *et al.,* 2004).

*P53* gene encodes a tumor suppressor protein which plays important role inside the cell especially in DNA transcription and repair, senescence, apoptosis, tumor suppression, treatment response and also response to changes in metabolism (Rivlin *et al.,* 2011). Protein domains represent independently folding units of protein with a size between 40 to 200 amino acids. Human *P53* protein contains three domains; transcriptional activation, DNA binding, and oligomerization domains. These domains are edged by a connecting regions. A proline-rich region links the transcriptional activation and DNA binding domains, a second proline-rich region links the DNA binding and oligomerization domains and a basic region forms the C-terminus of the protein (Stavridi *et al.,* 2005)

Mutations in the *P53* gene are quite frequent in esophageal carcinoma with a distinct pattern reported in tumors from high risk areas compared with low risk areas (Smeds *et al.* 2002).

There is now strong evidence that mutation not only abrogates p53 tumor-suppressive functions, but in some instances can also provide mutant

proteins with novel activities. Such neomorphic p53 proteins are capable of dramatically altering tumor cell behavior, primarily through their interactions with other cellular proteins and regulation of cancer cell transcriptional programs. Different missense mutations in *TP53* may confer unique activities and thereby offer insight into the mutagenic events that drive tumor progression (Freed-Pastor and Prives 2012).

In Sudan annually female have high incidence of esophageal cancer than males and the cases are jumped from 140 in 2000 to 341 in 2012. Most of the cases are comes from Khartoum, North Kordofan, and West Nile States. (RICK 2013).

Single nucleotide polymorphism (SNP) is a significant type of genetic variation commonly detected in human genome (Nadeau 2002). A total of 336845724 SNPs have been identified in humans and have been deposited in NCBI dbSNP; human P53 gene share of these SNPs 3115 SNPs. SNP arising in coding region may cause an amino acid change in the corresponding protein and in such case it is called as non-synonymous SNP (nsSNP) or may not change the amino acid and here it called synonymous SNP; these nsSNPs change the protein structure and hence its function, causing a specific disease (Ramensky *et al.* 2002; NCBI 2018)
There are recently a number of articles representing the association of SNPs in the TP53 gene with different cancers type, but in silico analysis has not yet been discussed on the functional, interactional and structural aspects of different types of SNPs in this gene. In the current study we used different bioinformatics prediction tools and databases for analysis of SNPs in TP53 gene.

Aloia *et al.* (2001) supports the prognostic value of immunohistochemical tumor markers, specifically the expression pattern of P53 in patients with EC treated with complete resection alone.

Taghavi *et al.* (2010) found that Over-expression of *P53* in association with cigarette smoking may play a critical role in esophageal squamous cell carcinoma (ESCC) carcinogenesis among high-risk population of north-eastern Iran.

Cao *et al.* (2004) suggest that the mutation and over expression of *P53* may play important roles in the development of ESCC. The changes in *P53* may reflect environmental exposure to the different combinations of mutagenic factors and genetic instability demonstrated by the populations in Linxian and Zhejiang in China.

The study of Shi *et al.* (1999) suggests that P53 mutation is an early event in esophageal carcinogenesis occurring in most of the dysplasia and carcinoma in situ lesions, and cells with such mutations will progress to carcinoma.

## 1.2. Rationale

The etiology of EC is a complex process that involves cumulative mutations in multiple genes, but its exact pathogenesis is still unclear. (Wang *et al.* 2012). In Sudan, researches that discuss esophageal cancer genetics using computational analysis seem to be limited. Therefore, this study is intended to discuss changes in *P53* gene, its functional impact and relationship with different demographic and histopathologic features like sex, age of the patients and type of the tumor in Sudanese patients. To the best of our knowledge, this research will be the first in this field in Sudan according to the published data.

## 1.3. Objectives

### 1.3.1. General objective

To sequence *TP53* Gene mutations exon 5-8 in Sudanese patients with esophageal carcinoma.

### 1.3.2. Specific objectives

To describe the histopathological features of EC in Sudanese patients.

To investigate *TP53* mutations in EC and correlate them with age, sex and tumor type.

To investigate the reliability of p53 accumulation as an indicator of p53 genetic alteration.

To compare the frequency and spectrum of *TP53* gene mutations in squamous cell carcinoma and adenocarcinomas of the esophagus.

# CHAPTER TWO

## LITERATURE REVIEW

# CHAPTER TWO

# LITERATURE REVIEW

## 2.1 Anatomy and histology of the esophagus:

The esophagus develops from the distal part of the primitive foregut. It is a hollow soft muscular tube approximately 10 inches (25 cm) long that runs from the oropharynx to the cardia of the stomach. It is located posterior to the trachea in the mediastinum of the thoracic cavity. After descending in the thoracic cavity, the esophagus penetrates the muscular diaphragm. A short section of the esophagus is present in the abdominal cavity before it terminates at the stomach. The normal esophagus is indented from above downwards by the arch of the aorta, the left bronchus and the left atrium (Ellis and Mahadevan 2013)

The wall of the esophagus consists of four layers: mucosa, submucosa, muscularis externa and adventitia. The mucosa is lined by stratified squamous epithelium with underlying lamina propria consisting of blood capillaries, lymphatics, loose connective tissue and lymphoid aggregates also has an immunologic role making up the mucosa-associated lymphoid tissue (MALT). In addition there is a thin muscularis mucosa of double layer smooth muscle (Gray and Standring 2008; Betts *et al.* 2014).

The submucosa lies immediately under the mucosa it is highly vascularized loose connective tissue containing mucous glands. The muscularis externa consists of skeletal muscle in the upper esophagus, skeletal and smooth muscle in the middle part and only smooth muscle in the lower esophagus. The outermost layer is the connective tissue adventitia. The cervical part of

the esophagus is supplied by the inferior thyroid artery, the thoracic part by bronchial and esophageal branches of the thoracic aorta and the abdominal part by ascending branches of the left phrenic and left gastric arteries (Gray and Standring 2008).

Lymphatic drainage of the upper third of the esophagus is via deep cervical lymph nodes, the middle third is drained by the superior and posterior mediastinal lymph nodes whereas the lower third drainage is through the left gastric and celiac nodes in the abdomen (Snell 2004).

The muscle of the esophageal wall is innervated by both autonomic and somatic nervous systems. The upper esophagus is supplied by branches of the recurrent laryngeal nerve and by postganglionic sympathetic fibers. The lower esophagus is supplied by the esophageal plexus, and contains a mixture of parasympathetic and sympathetic fibers. Motor fibers via the vagus and axons from neuronal cell bodies via the recurrent laryngeal nerve supply cricopharyngeus and the striated muscle of the upper third of the esophagus. Axons with cell bodies in the dorsal nucleus of the vagus pass through the esophageal plexus and supply the smooth muscle of the lower two-thirds of the esophagus. Vasomotor sympathetic fibers also innervate the vessels of the cervical and upper thoracic esophagus (Michael and Wojciech 2011; D'Antoni 2016).

## 2.2. Physiology of the esophagus:

The main function of the esophagus is to transport swallowed food into the stomach by peristalsis (Krause 2005; Allen 2008; Marieb 2015). When food enters the esophagus, the smooth muscle is stretched; this initiates a peristaltic wave that proceeds along the length of the esophagus, propelling

the food with it. When the peristaltic wave reaches the end of the esophagus, the smooth muscle at the opening into the stomach relaxes and food moves into the stomach. Another important function of the esophagus is to prevent gastroesophageal reflux. When a peristaltic wave is not passing down the esophagus, the esophageal sphincter is in the closed position, preventing reflux of stomach contents into the esophagus. Reflux is also prevented due to the presence of the lower esophageal sphincter is in the abdominal cavity. This minimizes the pressure difference between the high-pressure abdominal zone and the low-pressure thoracic area thus preventing the backflow of food (Corwin 2008).

## 2.3. Pathology of esophagus

Pathological conditions of the esophagus are variable and include malformations, inflammatory and non-inflammatory conditions and neoplasia.

### 2.3.1. Malformations

The esophagus develops from the distal part of the primitive foregut. From the floor of the foregut also differentiate the larynx and trachea. This close relationship between the origins of the esophagus and trachea accounts for some relatively common malformation such as: esophageal atresia with tracheoesophageal fistula or esophageal atresia without concomitant fistula and opening of the upper esophagus into the trachea. Rarely, tracheoesophageal fistula occurs without atresia (Ellis and Mahadevan 2013). Congenital strictures and congenital fibrosis at the gastro-esophageal junction (Schatzki ring) may be encountered (Mullick and Falk 2001).

### 2.3.2. Inflammation

Esophagitis or inflammation of the esophagus can be caused irritant chemicals m medications, physical factors, food allergies and infections e.g. bacteria and mycotic infections. Reflux of gastric juice from the stomach causes reflux esophagitis which may lead to the development of Barrett esophagus. The latter is characterized by metaplasia of the lining stratified squamous epithelia of the lower esophagus to simple columnar epithelia and may predispose to EC (Shaheen et al., 2009).

### 2.3.3. Circulatory disturbances

Esophageal varices are one of the important circulatory disturbances. They form as a consequence of portal hypertension, most often associated with alcoholic cirrhosis or hepatic schistosomiasis. Esophageal varices may suddenly rupture causing fatal bleeding, hematemesis and shock (WEST and MITCHELL 2009).

### 2.3.4. Motility disorders

Food motility can be affected by different disorders like difficulty in swallowing (dysphagia), or painful swallowing (odynophagia). In addition to Achalasia which is refers to a failure of the lower esophageal sphincter to relax appropriately. This leads to megaesophagus which is a progressive enlargement of the esophagus. Spasm of esophageal muscles may lead to chest pain and fibrosis can cause hardening of the esophageal walls and interfere with peristalsis (Walker and Colledge 2013; Purves *et al.* 2014).

Cancer causes thickening of the esophagus wall, narrowing of the lumen and dysphagia. Treatment of esophageal cancer by surgery, radiation therapy or

chemotherapy may have side effects which lead to swallowing problems, such as fibrosis, narrowing of the esophagus and infection (Cancer.Net 2017).

### 2.3.5. Esophageal tumors:

Esophageal tumors may be benign or malignant. Benign tumors, such as papilloma, adenoma, Leiomyoma and fibro-vascular polyp may arise from epithelial or mesenchymal elements. They are usually slow growing and asymptomatic found incidentally during routine endoscopy or radiography. They may cause swallowing problems but rarely ulcerate or bleed. Of these, leiomyoma is the most common benign tumor and usually has a favorable prognosis (Chou & Gress 2011).

Concerning the malignant type of EC, SCC and AC are the two morphologic variants that account for more than 95% of EC. SCC is more common worldwide especially in nonindustrial countries, but in the United States and other Western countries AC is on the rise (Goljan 2007; Kumar *et al.* 2013).

AC is the predominant EC in developed nations and is the most common primary cancer of the distal esophagus. Barrett's esophagus and long-standing gastroesophageal reflux disease (GERD) are the most common predisposing causes. Prevention of GERD and diets rich in fresh fruits and vegetables decrease the risk for developing AC but dysplasia, tobacco use, obesity, and previous radiation therapy are considered as risk factors (Kumar *et al.* 2013).

SCC is the most common primary cancer in developing countries and shows marked geographical variation in incidence. It is more common in black Americans than whites and is male dominant. Risk factors include smoking, alcohol abuse and Lye strictures, achalasia, Plummer-Vinson syndrome. It may be located in midesophagus (50% of cases) and lower esophagus. It spreads to local nodes first and then to liver and lungs. Dysphagia for solids, may be with weight loss, is the most common presenting symptom (Goljan 2007; Short *et al.* 2017).

## 2.4. Epidemiology of the esophageal cancer

EC is the eighth most common cancer worldwide, with an estimated 572,034 new cases in 2018 (3.2% of all sites), and the sixth most common cause of death from cancer with an estimated 508,585 deaths (5.3% of all sites) (Bray *et al.* 2018).

Incidence rates of EC vary internationally by nearly 16 fold, with the highest rates found in Southern and Eastern Africa and Eastern Asia and lowest rates observed in Western and Middle Africa and Central America in both males and females. EC is 3 to 4 times more common among males than females (Jemal *et al.* 2011).

Although SCC accounts for about 90% of cases of esophageal cancer worldwide, the incidence of and mortality rates associated with esophageal AC are rising and have surpassed those of esophageal SCC in several regions in North America and Europe. EC is rare in young people and increases in incidence with age, peaking in the seventh and eighth decades of life. AC is three to four times as common in men as it is in women, whereas the sex distribution is more equal for SCC (Rustgi & El-Serag, 2014).

EC is one of the least studied fatal cancers worldwide because of its extremely aggressive nature, poor prognosis and high mortality rate ranking sixth among all cancers (Zhang 2013).

Epidemiological studies in high-incidence areas have indicated that a high dietary intake of tannic acid, in the form of strong tea or sorghum wheat, or dietary deficiencies of riboflavin, vitamin A and possibly zinc may predispose to cancer, but other factors such as fungal contamination of foodstuffs, opium usage and thermal injury may also be involved. In Western countries, cigarette smoking and drinking alcoholic spirits are associated with a higher incidence. A factor of current interest is the possible involvement of HPV. Some ECs contain HPV in their cells, and viruses of similar subtype can be found in intact and apparently normal esophageal mucosa. It is therefore possible that virus integrated into the host genome can bring about oncogene activation and carcinogenesis. The involvement of papilloma viruses in the development of bovine esophageal carcinoma is well established (Underwood and Cross 2009).

Worldwide, regions with very high incidence have been identified in Iran, South Africa, Brazil and Central China. In Henan Province in China the mortality rate from EC exceeds 100 per 100 000 in males and 50 per 100 000 in females. The squamous epithelia show pleomorphism, disordered maturation with immature cells and mitotic activity (Underwood and Cross 2009).

By the 1990s, AC was the predominant type of esophageal cancer in the US, surpassing SCC. In 2014, there were approximately 18,170 incident EC in the US, 59.9% of which were AC (Rubenstein and Shaheen 2015).

In European countries, the annual incidence is around 5 per 100 000 population in males and 1 per 100 000 in females. However, there are some well-defined high-risk areas, such as North-West France and Northern Italy, where the incidence rises to 30 per 100 000 in males and 2 per 100 000 in females (Underwood and Cross 2009).

### 2.4.1 Mortality

Overall factors that were associated with an increased mortality risk included increasing age at diagnosis, black race versus white race, histologic grade, primary tumor site in the lower esophagus and abdomen versus upper regions, and increasing depth of invasion (Eloubeidi *et al.* 2002).

In previous studies, AC has had a more adverse effect on survival than SCC, and poorly differentiated carcinoma has had a more adverse effect than well or moderately differentiated carcinoma (Lieberman *et al.* 1995).

### 2.4.2 Survival

Poor outcome of esophageal cancer treatment remains a real problem. Regardless of improved treatment modalities recurrence is still considered a major problem even with improved staging and treatment options (Waraich *et al.* 2011). EC five year mortality exceeds 85% to 90% (Ilson 2008). Survival of patients with EC largely depends on disease stage and progression. More than 50% of EC patients have advanced, unresectable disease or present with distant metastases upon diagnosis, with an average of 8 to 10 months overall survival and a dismal 5%-17% 5-year survival rate (Zheng *et al.* 2016).

Patients who have EC experience very poor survival. Only 16% of patients in the USA and 9% in Europe live for 5 years after diagnosis. This is mostly because the diagnosis is late and after symptoms occur (Chava *et al.* 2012).

In a meta-analysis done by Fisher *et al*. they found that the patients with AC and TP53 gene mutations have reduced overall survival compared with patients without these mutations, and this effect is independent of tumor stage (Fisher *et al.* 2016).

The fact remains that, regardless of histology, EC has a poor prognosis: only about 1 in 5 patients survive 3 years or more beyond the date of diagnosis (Umar and Fleischer 2008).

Unfortunately, a substantial proportion are simply intubated to facilitate adequate nutrition. The long-term outlook is therefore very poor, with only a 5% survival at 5 years. Most patients die of local disease and bronchopneumonia exacerbated by malnutrition. Unlike many forms of cancer, metastases are rarely found at autopsy (Underwood and Cross 2009).

## 2.5. Preventive factors

Prevention of EC is of extreme importance given the limitations of screening for esophageal SCC and the minor percentage of patients who develop esophageal AC in the case of Barrett's esophagus. In order to prevent the disease we should take in account the risk factors and work on regular screening for conditions that can lead to esophageal cancer (Chung *et al.* 2015; Kim and Shah 2017)

In Western countries there is more incidence of AC cases which is considered one the most predisposing factors for Barrett’s esophagus (BE).

Eradication of predisposing factors one of the major step for primary prevention of both esophageal AC and SCC. Primary prevention include: Avoidance of meat and processed food, lower esophageal sphincter relaxing drugs, increasing fruits and vegetables consumption, looking after ideal body weight and lifestyle modification for GERD. In secondary prevention the recommended steps are; endoscopic screening white men aged over 50 with long term GERD symptoms approximately 5 years, proton pump inhibitors (PPI) therapy for GERD and BE. Tertiary prevention include regular endoscopic surveillance for Barrett's esophagus, Endoscopic treatment of high grade intraepithelial neoplasia/carcinoma in-situ HGIN/CIS. In eastern countries SCC more frequent than AC. The primary prevention in this case is the avoidance of alcohol consumption, cigarette smoking, betel quid chewing, meat, processed food, hot drinks, high fruits and vegetables intake and adequate oral hygiene. In secondary prevention; Endoscopic screening of high risk population with alcohol consumption, cigarette smoking, betel quid chewing and history of head and neck cancers. In tertiary prevention; endoscopic treatment of HGIN, high grade intraepithelial neoplasia; CIS, carcinoma in situ (Chung *et al.* 2015) .

## 2.6. Etiology of esophageal cancer

### 2.6.1. Risk factors for squamous carcinoma

In retrospective studies of EC, smoking, hot tea drinking, red meat consumption, poor oral health, low intake of fresh fruit and vegetables, and low socioeconomic status have been associated with a higher risk of esophageal SCC (Zhang 2013).

SCC is the most frequent histological type in black individuals and white women, while AC is predominant in white men the incidence of esophageal squamous cell carcinoma is generally higher in men than women in most countries. Smokers have a 5-fold risk of developing this disease compared to non-smokers, and alcohol intake relative risk (RR) increases with the amount of alcohol ingested varying between 1.8 and 7.4 depending on the weekly volume. The intake of certain types of drink creates worldwide "hot spots" of squamous cell carcinoma of the esophagus. Mate has been linked for both amount consumed and temperature. Also individuals who eat foods rich in nitrogenous components have higher risk compared to those who do not. Areca nut chewing sometime mixed with tobacco, have been related to the development of SCC. In addition to deficits of minerals and vitamins, due to low intake of fruits and vegetables (Wheeler and Reed 2012; Arnal *et al.* 2015)

Tylosis is an autosomal dominant disease that are clearly related to the development of esophageal squamous carcinoma. Familial aggregation in population of high incidence has also been reported. Four genome wide association studies (GWAS) conducted in Chinese and Japanese population have shown genetic susceptibility factors in the development of squamous carcinoma, especially in heavy alcohol and tobacco users (Zhang *et al.* 2012).

### 2.6.2. Risk factors for esophageal adenocarcinoma

Esophageal adenocarcinoma (EAC) is rapidly increasing in incidence in Western cultures. Barrett's esophagus (BE) is the presumed precursor lesion for this cancer. Several other risk factors for this cancer have been described,

including chronic heartburn, tobacco use, Caucasian race, and obesity. Despite these known associations, most patients with EAC present with symptoms of dysphagia from late-stage tumors only a small minority of patients are identified in screening and surveillance programs (Rubenstein and Shaheen 2015).

The incidence of esophageal adenocarcinoma is 8-fold more common in men than in women and 5-fold more common in whites than in blacks in the United States. The increased incidence of BE is correlated with an increased incidence of AC in the same period. BE develops in 6%-14% of patients with GERD and of which, around 0.5%-1% will develop AC. Detection of low-grade dysplasia was associated with an incidence rate for AC of 5.1 cases per 1000 person-years compared to 1.0 case per 1000 person-years among patients without dysplasia. These data question the rationale for ongoing surveillance in patients who have BE without dysplasia (Arnal *et al.* 2015).

Obesity is a major and consistent risk factor for the development of esophageal AC. A body mass index (BMI) $>$ 25 was associated with an increased risk of esophageal AC. Two main mechanisms have been proposed for the development of esophageal AC in obese patients. First, a physical mechanism involving an increase in the incidence of GERD, and second a hormonal-dependent mechanism mediated by inflammatory markers that are secreted by adipocytes (Renehan *et al.* 2008; Arnal *et al.* 2015).

Alcohol is not related to the presence of adenocarcinoma, but smoking tobacco is a known risk factor, with an OR of 2.7 (95% confidence interval

(CI): 1.64-4.45) relative to non-smokers. In a United States case-control study it was found that a diet rich in vitamins, fruits and vegetables protect against the development of this disease (Arnal *et al.* 2015).

Observational studies with a large number of patients showed that the use of non-steroidal anti-inflammatory drugs (NSAIDs), PPIs and statins in patients with BE, reduced the progression to adenocarcinoma (Arnal *et al.* 2015).

Genome-wide association study (GWAS) demonstrated that risk of BE and esophageal AC is influenced by many germline genetic variants of small effect and that shared polygenic effects contribute to the risk of these two diseases. Researchers found that the genetic correlation between BE and esophageal adenocarcinoma was high. These data strongly propose that shared genes underlie the development of BE and esophageal adenocarcinoma. The first GWAS of esophageal AC with BE suggesting that much of the genetic basis for esophageal AC lies in the development of BE, rather than it's to esophageal adenocarcinoma (Ek *et al.* 2013).

## 2.7. Signs and symptoms of esophageal cancer

Clinical Manifestations of EC include dysphagia which is one of the most common and earlier symptom, Anorexia followed by weight loss and pain from bone metastases often is the first symptom that stimulates a person to seek care (Corwin 2008).

Early cancers of the esophagus generally are asymptomatic, although ulcerated lesions may sometimes present with gastrointestinal bleeding, such as melena, or be found during workup for occult gastrointestinal bleeding or iron deficiency anemia. Advanced cancers can also cause bleeding, but most

commonly present with dysphagia. Cancers usually become quite large before compromising the lumen sufficiently to cause the sensation of blockage of a swallowed food. Difficulty ingesting solid foods occurs before liquids. Symptoms commonly have a very gradual onset. Weight loss is usually present. Odynophagia, the sensation of pain with swallowing is less common (Lightdale 1999).

Clinical Manifestations include: Dysphagia which is the most common symptom, Anorexia followed by weight loss and pain from bone metastases often is the first symptom that stimulates a person to seek care (Corwin 2008).

## 2.8. Diagnosis of esophageal cancer

Diagnostic Tools that help in the diagnosis of the disease are endoscopy followed by tissue biopsy which is considered the primary method for the diagnosis of esophageal carcinoma and X-ray or other diagnostic tests may be used to identify the secondary tumors(Lightdale 1999; Corwin 2008).

## 2.8.1 Laboratory investigations

### 2.8.1.1. Histopathology

Most of esophageal lesions were found to be AC and SCC, with an insignificant number of adenosquamous lesions and small cell carcinomas. Whereas the type of carcinoma may have little influence on prognosis in the majority of lesions, it may be better to have an adenocarcinoma they have less local recurrence and fewer new primary lesions. Irrespective of the prognostic implications, it provides useful validation of the presurgical

diagnosis, which may be important in adjuvant therapy decisions (RCPath Cancer Services Working Group 2013).

#### 2.8.1.2. IHC markers

In a systematic review done by Wang et al. on immunohistochemical prognostic markers of ESCC they identified eleven IHC markers with reproducible results, including eight markers indicating unfavorable prognosis [epidermal growth factor receptor (EGFR), Cyclin D1, vascular endothelial growth factor (VEGF), Survivin, Podoplanin, Fascin, phosphorylated mammalian target of rapamycin (p-mTOR), and pyruvate kinase M2 (PKM2)] and 3 markers indicating favorable prognosis of ESCC (P27, P16, and E-cadherin)(Wang *et al.* 2017) .

#### 2.8.1.3. Inflammatory markers

In a study done by Hardikar *et al*. they found that C-reactive protein (CRP) level above the median was associated with 80% increased risk of esophageal AC. Persons with interleukin 6 (IL-6) levels above the median also had almost twofold increased risk. Concentrations of tumor necrosis factor (TNF) receptors and F2-isoprostanes were not associated with AC risk (Hardikar *et al.* 2014).

In a study done on squamous cell esophageal carcinoma patients. The CRP level was measured prior to and following the completion of neoadjuvant Radiochemotherapy (RCT). CRP levels were high prior to treatment; however, eventually decreased and normalized following the therapy. In univariate analysis, pre-therapeutic CRP levels had a significant influence on the response rate, whilst post-therapeutic CRP levels had no significant influence. And no association was observed between CRP levels and

survival. This preliminary data indicated that the pre-therapeutic serum CRP level is a possible indicator of treatment response to RCT (Badakhshi *et al.* 2016).

A total of 283 patients 201 with EAC and 82 were found to have ESCC with locally advanced EC were enrolled in a study done by Jomrich *et al*. 167 patients received neoadjuvant treatment (59.0%). Simple analysis revealed that there were significant differences in cancer specific survival in relation to elevated CRP, lymph node status (Jomrich *et al.* 2017).

#### 2.8.1.4. Molecular Diagnosis

##### 2.8.1.4.1. DNA extraction

Extracting DNA from (FFPE) tissue still considered as a challenge, great effort has been made to improve the available methods for extracting DNA from FFPE samples with higher quality (Lin *et al.* 2009).

##### 2.8.1.4.2. Polymerase chain reaction

The polymerase chain reaction (PCR) revolutionized the field of molecular biology. many copies can be generated from very small amount of DNA for advanced analysis (Boyle 2014). This copying process consist of different steps include consecutive cycles of heating and cooling of samples in a thermal cycler machine for more than 30 cycles (Butler 2011). This process lead to production of a considerable amounts of DNA sufficient for a variety of analyses, including subsequent sequencing analysis (Boyle 2014).

#### 2.8.1.4.3. DNA sequencing

DNA sequencing is the technique used to determine the nucleotides sequence within a DNA. Knowledge of DNA sequences has become an important tool in different research aspects and has applications in wide range of scientific fields. DNA sequencing was first introduced in mid-fifties by Fred Sanger, to determine insulin molecule sequence (Pham 2018).

### 2.8.2. Imaging techniques

Most patients with esophageal cancer present with dysphagia, which is typically a symptom of advanced disease. Barium studies are often used to evaluate these patients and double contrast barium studies have been found to be sensitive for the detection of carcinoma of the esophagus and esophagogastric junction with a positive predictive value of 42% (Iyer and Dubrow 2004).

Computed tomography (CT) is considered complimentary to endoscopy and barium studies and is used to stage and follow esophageal tumors. CT can be used to define the local extent of tumor by showing the extent of involvement of the esophageal wall by tumor and tumor invasion of the peri-esophageal fat. CT cannot reliably delineate the individual layers of the esophageal wall and therefore cannot distinguish between T1 and T2 lesions. Infiltration of the tumor into the peri-esophageal fat as seen on CT denotes a T3 tumor and does adversely affect prognosis, although en-bloc resection for cure may still be attempted (Saunders *et al.* 1997; Iyer and Dubrow 2004).

Magnetic resonance imaging (MRI) provides little advantage over CT in staging esophageal tumors. MRI also cannot reliably distinguish the

different layers of the esophageal wall, which is crucial for accurate local staging. Nodal disease and distant metastases can be shown by CT or MRI (Iyer and Dubrow 2004).

## 2.9. Treatment

### 2.9.1. Surgery

The surgical approach used for esophageal resection depends on tumor characteristics and location, the surgeon's training and experience, and overall surgical philosophy. The most popular approaches. In the United States are transthoracic and transhiatal. There are purported advantages and disadvantages to each approach. The transthoracic technique permits direct visualization of the tumor and dissection of more periesophageal and nodal tissue. The disadvantage of this operation is the cardiopulmonary insult imposed by a thoracotomy and the increased morbidity of anastomotic leak in the thorax. The transhiatal approach has the potential to minimize respiratory compromise, and anastomotic complications are usually easily managed. Its disadvantages are the inability to perform a complete node dissection or to always completely visualize the tumor (Reed 1999).

### 2.9.2. Radiation therapy

#### 2.9.2.1. Radiation Alone

When using radiation therapy alone that results in poor local control and poor survival. Local recurrence rates range from 52% to 77% with standard fractionation. Five-year overall survival rates range from 0% to 21%. In the largest review, which comprised 49 series and included 8,500 patients with esophageal cancer treated with radiation therapy alone, 1, 2 and 5 year

overall survival (OS) rates were 18%, 8%, and 6%, respectively (Shridhar *et al.* 2013).

#### 2.9.2.1.1. Preoperative Radiation

There is several randomized studies have confirmed the validity of concurrent chemoradiotherapy to radiotherapy alone for surgically inoperable patients (Araújo *et al.* 1991; Herskovic *et al.* 1992; Kolaric *et al.* 1992; Al2sarraf *et al.* 1997).

In a trial conducted in India 1999, they found both surgery and radiotherapy can improve the quality of swallowing significantly for patients with operable esophageal carcinoma. Surgery is slightly superior to radiotherapy in improving the quality of swallowing. Survival in the surgery arm was significantly better than that in the radiotherapy arm (Badwe *et al.* 1999).

Many randomized trials were conducted to lower total doses and shorter intervals from end of radiation to surgery compared with modern treatment regimens. The 5-year OS rates ranged from 9% to 30% for surgery alone and 9% to 45% for radiation and surgery. Time to surgery also differ, ranging from 8 days or less to 2 to 4 weeks. A meta-analysis of randomized trials was conducted to assess the benefit of neoadjuvant radiation compared with surgery alone. There was increased survival with preoperative radiation (Shridhar *et al.* 2013).

#### 2.9.2.1.2. Postoperative Radiation

The Japanese Esophageal Oncology Group (JEOG) evaluated postoperative radiotherapy versus chemotherapy. Overall survival was no different; 3-year survival rates were 51% in radiation therapy and 52% in chemotherapy and

local recurrence rates were also equivalent. On the other hand in a retrospective study conducted by Chen et al of patients with SCC of the mid-thoracic esophagus, local lymph node recurrence rates were considerably increased with adjuvant radiation therapy compared to chemotherapy or no adjuvant therapy group 20.4%, 32% and 42.7% respectively. In conclusion, postoperative radiation alone should not be considered as a therapeutic choice (OncologyGroup 1993; Chen *et al.* 2009).

#### 2.9.2.2. Adjuvant and Neoadjuvant Chemoradiation

Adjuvant therapy for cancer usually refers to surgery followed by chemo or radiotherapy to help decrease the risk of the cancer recurrence. In patients with node-positive AC of the gastroesophageal junction, adjuvant chemoradiotherapy is the standard approach (Shridhar et al. 2013; MFMER 2015).

Radiochemotherapy is the treatment of choice for nonsurgical patients, with survival rates similar to those after surgery alone. Persistent disease after synchronized radiochemotherapy indicate a locoregional failure. Neoadjuvant Chemoradiation which involve adding surgery to definitive concurrent radiochemotherapy has increased locoregional control without a detectable benefit for overall survival rate. It has been shown to downstage tumors and increase the complete resection rate. However, this treatment method remains uncertain because there is no survival benefit recognized with such an invasive treatment (Liao *et al.* 2007).

#### 2.9.2.3. Esophageal cancer and radiation therapy side effects

Side effects of radiation therapy for esophageal cancer include: Skin reactions which include redness, dryness, itchiness and darkening or

thickening; fatigue triggered by anemia, poor appetite or depression and toxic substances from cancer cells break down; nausea and vomiting which is occur a few hours after radiation treatment or chemoradiation; xerostomia occurs within the first 2–3 weeks of treatment and can become worse as treatment goes on. Radiation also can cause pharyngitis or esophagitis, which can make swallowing difficult, it's usually start 2–3 weeks after radiation treatment starts. A stomatitis or oral mucositis may occur if radiation therapy includes the mouth or after chemoradiation. A sore throat may occur if radiation is given to the upper part of the chest, symptoms improve a few weeks after treatment is completed. Diarrhea it may occur if upper abdomen is being treated. Diarrhea usually goes away once radiation therapy treatments are done. Loss of appetite which can lead to weight loss and malnutrition. Furthermore narrowing of the esophagus which is related to esophagitis. Also pulmonary, cardiac, and mucosal toxicities happens especially after radiochemotherapy. Moreover fistulas may develop between the esophagus and trachea in people treated with radiation therapy for esophageal cancer. This may cause some food leak into the trachea, which can lead to infection and choking (Liao *et al.* 2007; Society 2018).

### 2.9.3. Endoscopic treatments

#### 2.9.3.1. Endoscopic mucosal resection EMR

EMR has been used since 1955. And involves a submucosal injection/lift of the lesion to create a fluid cushion that creates a safety margin for cautery and cutting. Advantages of EMR include simplicity of the technique, safety, larger samples can be obtained in contrast to biopsies. Limitations include a

higher recurrence rate and lower rates of *en bloc* resection than Endoscopic submucosal dissection (ESD) provides (Balmadrid and Hwang 2015).

#### 2.9.3.2. Endoscopic submucosal dissection ESD

ESD technique arose from the high incidence of gastric cancer in Asian populations to reduce mortality from cancer, ESD was perfected in these countries and applied to different parts of the gastrointestinal (GI) tract, such as the esophagus. ESD is a challenging technique that involves creating a large submucosal cushion through submucosal injections, and through the use of various cautery needle knife devices, cutting the lesion out in one piece. Extensive training and appropriate numbers of procedures are important in mastering this technique. Difficulty of visualization and positioning in addition to bleeding happens during the procedure. Complication rates and total procedure time will be higher at the start, but decrease with increased procedure volume and experience. In general, curative resection and recurrence rates are better in contrast to conventional EMR (Balmadrid and Hwang 2015).

### 2.9.4. Chemotherapy

Chemotherapy can be introduced at different times during treatment of EC. Adjuvant chemotherapy is a type of chemotherapy that can be given after surgery. The goal is to kill any cancer cells that may have been left behind during surgery, as well as cancer cells that might have escaped from the primary tumor and spread into other parts of the body. Another type is neoadjuvant chemotherapy which is a type of chemotherapy given before surgery often with radiation in order to shrink the tumor and make surgery easier. Finally there is chemotherapy for advanced cancers; it used for

metastasized cancers that have been spread to other organs, such as the liver, lung or bone it helps minimize tumor size and relieve symptoms of the disease. The goal here is not to cure the cancer but to extend overall survival (Society 2017).

#### 2.9.4.1. Chemo-drugs used to treat esophageal cancer

There are common drugs and drug combinations used to treat esophageal cancer. Older single agents includes bleomycin, 5-fluorouracil (5-FU) cisplatin, and mitomycin carboplatin. In addition to taxanes and other new agents. taxanes are a class of anticancer their mode of action depends on binding to tubulins/microtubules which suppress the microtubule dynamics and results in the blockade of cell mitosis, and eventually apoptosis. Examples of this type is oxaliplatin, paclitaxel and topoisomerase I inhibitor etoposide. Finally there is combination chemotherapy like Cisplatin and 5-FU, Epirubicin/Cisplatin/5-FU and Paclitaxel/Cisplatin/5-FU (Ilson 2008; Ojima *et al.* 2016).

### 2.9.5. Targeted therapy

For esophageal squamous cell carcinoma and gastro-esophageal adenocarcinoma, possible tumor targets have been described and many targeted therapies for esophageal cancer are in various phase I and II clinical trials. In esophageal cancer, novel targeted treatments are in early development, although encouraging results have been reported with antibodies directed at the EGFR and VEGF ligand, as well as with the oral TKIs. In the near future, new research trials will expand our treatment options significantly (Tew *et al.* 2005).

### 2.9.6. Immunotherapy

Also referred to as biotherapy. Complete remissions can be achieved by inducing an immune response against a number of cancers. This type of treatment have fewer side effects than other cancer management methods because of its mechanism of action, and is a potential option for peoples with cancer that is resistant to chemotherapy and/or radiation (Chen 2018).

Esophageal squamous cell carcinoma have been frustrating to treat, with slow progress made on extending survival. Immunotherapy targeting immune checkpoints, T cells, and infiltrating lymphocytes has shown promise in early studies. The efficacy of pembrolizumab and nivolumab is encouraging. Anti-chemokine receptors and oncolytic viruses are also making headway against these stubborn tumors; improved results when immune checkpoint inhibitors are combined with radiation therapy are eagerly anticipated. Adoptive T cell therapy and vaccines are also under development (Kojima and Doi 2017).

## 2.10. Complication of esophageal cancer

Dysphagia, difficulty or discomfort in swallowing, is one of the most common complication of EC among patients with unresectable disease. There are five levels of dysphagia, starting from the ability to eat a normal diet to some solids, semisolids, liquids and to complete dysphagia (Dai *et al.* 2014).

Tracheoesophageal fistulas also is another EC complications which is an abnormal opening that arises between the trachea and the esophagus due to the close anatomical relationship; in this case advanced esophageal cancer

can directly invade into the respiratory tract. As a result oral feeding is compromised and the quality of life in these patients is significantly reduced. Treatment options of tracheobronchial fistula includes both placing of endoscopic stent and surgical bypass (Kimura *et al.* 2015).

Anemia is a frequent finding in cancer patients, occurring in >40% of cases. In patients treated with chemotherapy, the incidence of anemia may rise to 90%. Anemia exerts a negative influence on the quality of life of cancer patients as it may contribute to cancer-induced fatigue. Anemia has also been identified as an adverse prognostic factor (Dicato *et al.* 2010).

Weight loss in patients with esophageal cancer occurs as a result of dysphagia, leading to reduced food intake, increased energy consumption caused by systemic inflammation induced by the tumor which enhances weight loss. This systemic inflammation results from local effects of the tumor directly or as secondary host response to tumor tissue necrosis and hypoxia. Thus, unintentional weight loss will occur in approximately 80% of all patients with esophageal cancer before diagnosis (van der Schaaf *et al.* 2014).

Metastasis is one of the common complications of EC. However, by the time most patients present with symptoms a direct spread to adjacent and distant organs has occurred. The most common areas of esophageal cancer metastases is lymph nodes, liver, lung, bones, adrenal glands, and brain in addition to other less common areas in the body (Underwood and Cross 2009; Shaheen *et al.* 2017).

## 2.11. Bioinformatics tools used in mutational analysis:

### 2.11.1. FinchTV

FinchTV (https://digitalworldbiology.com/FinchTV) shows base calls and the chromatograms displays quality values above the trace, and allow to edit bases and save the edited file, download the file in (FASTA format) for analysis in other computer packages, and to perform a BLAST search one sequence, It also allows adjustment of vertical and horizontal scales and provides with the ability to print out trace all on one page in Portable Document Format (PDF) format (Leicester 2012; Bio-Rad [n.d.]).

### 2.11.2. CodonCode aligner

Sequences were assembled into contigs end clipped and edited using CodonCode Aligner software 8.0.1 (www.codoncode.com) (Dedham, MA).

### 2.11.3. BioEdite

BioEdit (http://www.mbio.ncsu.edu/BioEdit/bioedit.html) is a biological sequence alignment editor makes alignment and management of sequences relatively easy on computer. The program allows shaded alignment figures and automated ClustalW alignment (Theraputics 2005).

### 2.11.4. SIFT Program

SIFT (Sorting Intolerant from Tolerant) tool (http://sift.bii.a-star.edu.sg/) uses sequence homology to calculate the probability of affecting protein function in case of amino acid change. It uses the concept of evolutionarily conserved regions which is less tolerant to mutations, and therefore amino acid change or frame shift mutations in these regions are expected to affect protein function the most. SIFT tool works by introducing a query protein

into SIFT program to be searched against protein database and aligned with homologous protein sequences. Then the program calculate SIFT score based on amino acid changes in that position. A SIFT score ranges from 0 to 1. Score less than 0.05 is predicted to affect protein function and considered functionally deleterious, whereas any score more than or equal to 0.05 represents a neutral substitution(Sim *et al.* 2012; Capriotti *et al.* 2013; Hepp *et al.* 2015)

### 2.11.5. PolyPhen -2

PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/) (Polymorphism Phenotyping v2) is a structural and functional predicting tool that predicts the effect of amino acid change on protein characteristics based on SNPs functional annotations, protein structural properties with sequence annotation, and finally predicst if the coding nonsynonymous SNPs is considered damaging or not (Adzhubei *et al.* 2010; Adzhubei *et al.* 2013).

PolyPhen-2 workflow requires protein sequence, mutational position and substitution. The PolyPhen output is represented with a score that ranges from 0 to 1, with zero score indicating a neutral effect of amino acid substitutions on protein function and a higher scores representing a mutation that is more likely to be damaging (Jia *et al.* 2014).

### 2..11.6. I-Mutant 3.0

I-Mutant 3.0 (http://gpcr2.biocomp.unibo.it/cgi/predictors/I-Mutant3.0/I-Mutant3.0.cgi) is a support vector machine (SVM) based tool, which was used to calculate the stability changes of specific SNP upon protein sequence. Information of wild and mutated residue, protein sequence,

temperature and pH were used as input parameters to this server, and finally the outputs reports if a point mutation is stable or not. The program categorizes the prediction into: neutral mutation (DDG = 0.5 kcal/mol), large decrease of stability (<− 0.5 kcal/mol) and large increase of stability (> 0.5 kcal/mol). The output is a free Gibbs energy change value (ΔΔG) of protein before and after mutation (Capriotti *et al.* 2005; Doss *et al.* 2012)

### 2.11.7. PhD-SNP

PhD-SNP (Predictor of Human Deleterious Single Nucleotide Polymorphisms) software (http://snps.biofold.org/phd-snp/phd-snp.html) is a prediction tool that predict disease association of nsSNP by dividing those SNPs into disease related or neutral polymorphism based on a score ranged from (0-1); SNPs with score above 0.5 are considered disease associated according to the program algorithm. PhD-SNP outputs depend on a number of sequences aligned, conservation index of SNP position, and frequencies of wild and mutant residues (Capriotti *et al.* 2013; Hepp *et al.* 2015)

### 2.11.8. Structural analysis using Project HOPE

Structural and biochemical analysis for mutations was accomplished using Project HOPE. HOPE (http://www.cmbi.ru.nl/hope/home) is a web-server used to give a comprehensive report on the effect of the specific mutation on the 3D structure of the protein using different software and sources. The user can submit a protein sequence or an accession number of specific protein after specifying the wild type residue and the new mutant form to create the report.

### 2.11.9. MutationTaster

MutationTaster (http://www.mutationtaster.org/) calculates the pathogenic consequences of variations in DNA sequence. It predicts the functional impact of amino acid alterations, intronic and synonymous substitutions, in addition to INDEL mutations and variants covering intron-exon connection region. Mutation Taster prediction system divides alterations as; disease causing: which is probably deleterious, disease causing automatic: the alteration here is known to be deleterious, polymorphism: probably harmless alteration and polymorphism automatic: known to be harmless (Schwarz *et al.* 2014; Schwarz and Seelow [n.d.]).

### 2.11.10. Catalogue of somatic mutation in cancer (COSMIC)

The COSMIC (Catalogue of Somatic Mutations in Cancer) (https://cancer.sanger.ac.uk/cosmic) database have been established to store somatic mutation data in one place and display all information related to human cancer (Bamford *et al.* 2004).

### 2.11.11. FATHMM

FATHMM (http://fathmm.biocompute.org.uk/) (Functional Analysis Through Hidden Markov Models) is a web-server predicts the functional significances of both coding and non-coding variants. We selected the cancer option to display predictions that can distinguish between cancer promoting/driver mutations and other germline polymorphisms. It uses a default prediction threshold of -0.75. Predictions with scores less than this indicate that the mutation is potentially cancer associated.

# CHAPTER THREE

## MATERIALS AND METHODS

# CHAPTER THREE

# MATERIALS AND METHODS

## 3. 1. Type of the study

This descriptive retrospective hospital based study was conducted during the period from July 2013 to July 2018 in different hospitals and medical centers in Khartoum State including: The National Health Laboratory, Ibn Sina Teaching Hospitals, National Radioisotope and Radiotherapy Center, Omdurman Teaching Hospitals, Khartoum Teaching Hospital, Alribat National Hospital, Alrrahma laboratory, Bahry Teaching Hospital, Military Hospital, Total lab Care Laboratory, Alalim Laboratory and Soba Teaching Hospital.

## 3.2. Study area

The study has been carried out in Khartoum State which lies between longitudes 31.5 to 34 °E and latitudes 15 to 16 °N. Khartoum State comprises three localities with total area of about 22,142 km2 and population of 7,687,500 people (Geohive 2008; Central Bureau of Statistics Sudan 2017).

## 3. 3 Study Samples

Patients Samples diagnosed with EC regardless of sex and age were included in this study. Patient diagnosed with other upper digestive tract problems were excluded.

## 3. 4. Samples (Materials)

A total of 204 tissue blocks belonging to EC patients were obtained from the histopathology departments of different hospitals mentioned above after all

ethical considerations have been fulfilled; the samples pertain to the period 2013–2017. Information about patients such as sex, age were also obtained. The sample size was calculated according to the internet sample size calculator based on the following data; esophageal cancer incidence rate between Sudanese population of (rate = 5.8 per 100,000), confidence level of 95% and 5% margin of error (SurveyMonkey 1999; Saeed *et al.* 2014).

### 3.5. Samples processing

The 204 biopsy samples collected were used for histopathology, immunohistochemistry and DNA extraction for molecular studies.

Two serial sections 4 µm thick were prepared from each paraffin block for histopathology and immunohistochemistry using a rotary microtome. Similarly, 3-4 sections 10 µm thick were cut for molecular study. Sections for histopathology were stained with hematoxylin and eosin as described by (Suvarna *et al.* 2012).

The stained sections were initially examined twice by different pathologist form different hospitals and then confirmed by a single pathologist and the results briefly described and categorized as normal esophageal histology, dysplastic changes, AC, and SCC. The slides were once more examined under Binocular light microscope, Olympus Optical Co. Ltd, Japan), adequately described and photographed using Sony digital camera, Model DSC W30, Japan.

### 3.6. Immunohistochemistry protocol:

Immunohistochemical staining was performed on 4 µm paraffin sections prepared from biopsy samples using primary antibodies (Rabbit monoclonal antibody, code CMC45331010) from CELL MARQUE, SIGMA-ALDRICH company, against P53. The sections were mounted onto poly-Llysine-

coated slides (Dako). Positive and negative controls were run in parallel with test slides, and included known positive p53 tissue section and negative staining tissues controls with a test sections processed with omitted step of primary antibody (reagent controls), and histologically matching the normal esophageal tissue. Following deparaffinization in xylene, slides were rehydrated through a graded series of alcohol and were placed in distilled water. Antigen retrieval was performed by boiling slides in antigen retrieval solution (1 mM citrate buffer) (pH 6.0), by 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, and After washing with PBS for 3 min then slides were incubated with (100 μ L) of monoclonal rabbit antihuman, against p53 for 30 min at room temperature in a humidity chamber. Primary anti-p53 antibodies identifying both mutant and wild-type p53. After washing with PBS for 3 min, binding of antibodies detected by incubating for 20 min with dextrin labeled polymer (Dako). Finally, the sections were washed in three changes of PBS, followed by adding 3, 3 di amino benzidine tetra hydrochloride (DAB) as a chromogen to produce the characteristic brown stain for the visualization of the antibody/enzyme complex for 5 min. After washing with distal water for 3 min. slides were counterstained with Mayer's haematoxylin for one min were washed in running tap water for 10 minutes (bluing), then dehydrate and, cleaned, mount in distyrene plasticizer xylene (DPX). Each tissue sections were evaluated independently by two investigators. A sample was defined positive when 10% of the neoplastic cells demonstrated nuclear staining (Cao, *et al*. 2004).

## 3.7. Molecular genetics:

### 3.7.1. Extraction of DNA from paraffin-embedded samples:

Genomic DNA for PCR analysis was extracted from FFPE tissue blocks by commercial DNA extraction kits (blackPREP FFPE DNA Kit, Analytikjena, Germany). Three to four (10-15 µm each) sections were cut from each paraffin block and put into 2.0 ml reaction tubes. New pair of gloves was used for each block. The chuck, forceps and knife holder were cleaned with xylene and rinsed with alcohol between sectioning of the blocks. To avoid contamination, sections from each block were cut using a new or unused area of microtome blade.

### 3.7.2. DNA extraction procedure:

Sections of FFPE material was put into 1.5 ml reaction tube and centrifuged at maximum speed for 1 minute. 400 µl Lysis Solution MA and 40 µl Proteinase K were added to the sample in the reaction tube and mixed vigorously by vortex for 10 seconds. The reaction tube was incubated at 65 °C for 1 hour in an incubator under continuous shaking at 1,000 revolutions per minute (rpm), to help in lysis of the sample. After lysis, the sample was placed into an incubator pre-heated to 90 °C and incubated for 1 hour under continuous shaking at 1,000 rpm. The sample was then incubated at room temperature for 5 minutes. The sample was centrifuged at maximum speed for 2 minutes and then transferred into a new 2.0 ml reaction tube avoiding carryover of residual FFPE material. 400 µl of absolute ethanol (96-99 %) was added to the sample, mixed vigorously by vortex for 10 seconds. The sample was then applied onto a Spin Filter located in a 2.0 ml Receiver Tube. After closing the cap the tube was centrifuged at 12,000 rpm for 1 minute. The Spin Filter was opened and 500 µl Washing Solution C was

added and after closing the cap centrifuged at 12,000 rpm for 1 minute. The Receiver tube with the filtrate was discarded and the Spin Filter placed into a new 2.0 ml Receiver Tube. Step 9 was repeated but using 650 µl Washing Solution BS and the Spin Filter placed into a new 2.0 ml Receiver Tube. Step 9 was again repeated but adding 650 µl ethanol absolute (96-99 %) and the Spin Filter placed into a new 2.0 ml Receiver Tube. The receiver tube was then centrifuged at maximum speed for 3 min to remove all traces of ethanol and then discarded. The Spin Filter was subsequently placed into a 1.5 ml Elution Tube and 100 µl Elution Buffer added. After incubation at room temperature for 2 minutes it was centrifuged at 12,000 rpm for 1 minute (Analytic Jena 2017). Extracted DNA was frozen in 1.5 ml tubes at -20°C until PCR detection of *TP53* exon 5-8 commenced.

#### 3.7.3. Polymerase chain reaction (PCR)

Four pairs of primers were used to amplify exon 5-8 DNA sequences of P53 gene. 2-5 µl of genomic DNA in 50 µl final reaction volumes was amplified using PCR protocol as follow: amplification conditions were; 95°C for 5 min, followed by 37 cycles at 95°C for 45 sec, primer-specific annealing temperature for 45 sec, 72°C for 45 second a final extension at 72°C for 5 min. 5 µl of the PCR products were applied on 2 % agarose gel and remaining PCR products were sequenced directly.

#### 3.7.4. Gel-electrophoresis:

The PCR products were visualized in 2% Agarose gel with Ethidium bromide staining 0.5 µg/ml working solution. The gel was prepared by dissolving 2 gm of agarose powder in 100 ml of 1X TBE buffer and heated at microwave oven for 60 sec until the agarose completely dissolved and clear, then left to cool at room temperature, one µl ethidium bromides was

added. The comb was then placed appropriately in the electrophoresis tray and then gel was slowly poured to avoid bubble formation and left to settle down for 30 min for solidification. 4 µl of 50 bp DNA ladder and 5 µl PCR product was loaded on the gel. Gel electrophoresis was performed at 100V and 36 Am for 30 minutes. Final results were viewed using UV Light gel visualization machine.

### 3.7.5. Interpretation of PCR results

PCR product length for exon 5 is 184 bp, exon 6, 128 bp, exon 7, 117 bp and 141 bp for exon 8 as shown in (Table.1).

**Table 1**. *P53*gene primers set.

| Exon | Sequence | A. Temp. | P. Size |
|---|---|---|---|
| **Exon 5:** | Forward: 5'-TAC TCC CCT GCC CTC AAC AA-3' | 59.7 ºC | 184 |
| | Reverse: 5'-CAT CGC TAT CTG AGC AGC GC-3' | | |
| **Exon 6:** | Forward: 5’-TTG CTC TTA GGT CTG GCC CC-3’ | 58.2 ºC | 128 |
| | Reverse: 5’-CAG ACC TCA GGC GGC TCA TA-3’ | | |
| **Exon 7:** | Forward: 5’-TAG GTT GGC TCT GAC TGT ACC-3’ | 59.3 ºC | 117 |
| | Reverse: 5’-TGA CCT GGA GTC TTC CAG TGT-3’ | | |
| **Exon 8:** | Forward: 5’-AGT GGT AAT CTA CTG GGA CGG-3’ | 53.8 ºC | 141 |
| | Reverse: 5’-ACC TCG CTT AGT GCT CCC TG-3’ | | |

### 3.8. Tools and web servers used in bioinformatics analysis of DNA sequences

The SNP information (SNP ID, amino acid position, mRNA accession number NM_000546.5, and protein accession number NP_000537.3) of the human P53 gene used in computational analyses was retrieved from Ensembl genome browser and the National Center for Biotechnology Information (NCBI) database of SNPs (http://www.ncbi.nlm.nih.gov/snp/).

The nucleotide and amino acid sequence of TP53 protein was obtained and investigated using nucleotide database NCBI, Basic Local Alignment Search Tool (BLAST) and UniProt Protein Database (http://www.uniprot.org). Sequence data are available at GenBank under accession numbers EU078744 to EU078840.

### 3.9. Statistical analysis

Data were analyzed using statistical package for social science software (SPSS software v. 21.0), frequencies were calculated. The results were evaluated using chi squire test and correlation and the differences were considered statistically significant when P. value $< 0.05$.

### 3.10. Ethical considerations

The study was approved by Sudan University of Science and Technology ethics committee. (No: DSR-IEC-13-05). Patients consent cannot be obtained because most of the patients were dead and the rest cannot be traced due to lack of contact data. Therefore all samples and medical data used in this study have been irreversibly anonymized to ensure patients privacy.

# CHAPTER FOUR

## RESULTS

# CHAPTER FOUR

# RESULTS

## 4.1. H&E results:

Two hundred and four esophageal blocks taken in different hospitals were diagnosed as esophageal carcinoma. The commonly diagnosed type was squamous cell carcinoma (SCC) amounting to 94.2 while adenocarcinoma (AC) constituted 5.8% of the cases; ratio: 16.2:1.0. The SCC was categorized as well differentiated (18.5%), moderately differentiated (20.4%) and poorly differentiated (9.0%) SCC. 41.2% were diagnosed only as SCC (Table 2). Five cases (2.4%) were described as invasive carcinoma (Fig. 1) and one case (0.5%) as in-situ carcinoma. Twenty two percent of the tumors showed desmoplasia. Epithelial pearls were frequent in well differentiated tumors. 25.2% of the sections were positive for IHC.

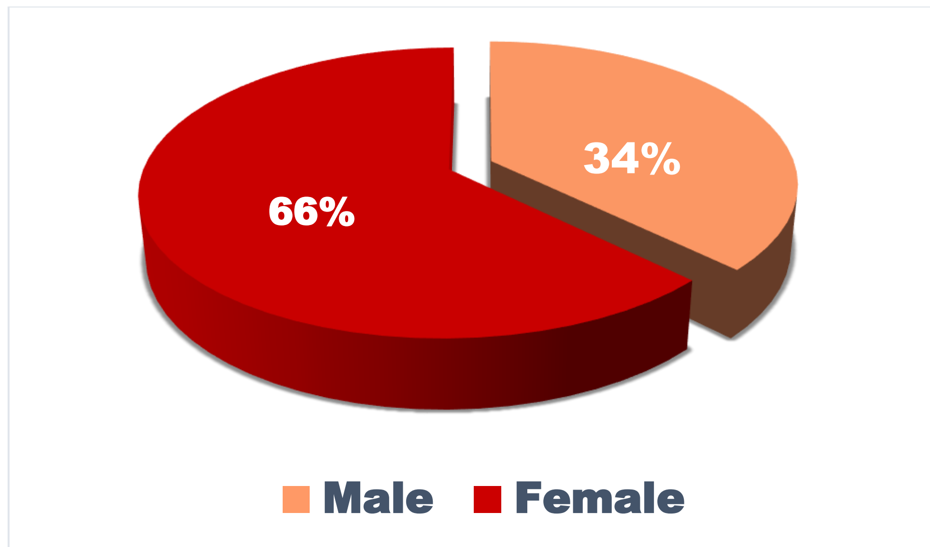



**Fig.1.** Percentage of male and female of EC patients.

**Table 2. Histopathological classification of esophageal carcinoma.**

| Histopathological findings | subtypes | percentage | Histopathologic type % | Total % |
|---|---|---|---|---|
| **Adenocarcinoma (AC)** | Not specified (AC) | 12/21 (5.9%) | 10.4 % | 100% |
| | Well differentiated (AC) | 1/21 (0.5%) | | |
| | Moderately differentiated (AC) | 5/21 (2.5%) | | |
| | Poorly differentiated (AC) | 3/21 (1.5%) | | |
| **Squamous cell carcinoma (SCC)** | Well differentiated (SCC) | 36/170 (17.6%) | 83.3% | |
| | Moderately differentiated (SCC) | 42/170 (20.6%) | | |
| | Poorly differentiated (SCC) | 9/170 (4.4%) | | |
| | Not specified (SCC) | 83/170 (40.7) | | |
| **Undifferentiated carcinoma** | Undifferentiated carcinoma | 13 (6.4%) | 6.4% | |

## 4.2. Immunohistochemical staining results:

Sections of EC positive for IHC staining showed diffuse brown nuclear staining (Fig. 48 a & b) while negative sections no or faint scattered nuclear staining was seen (Fig. 49 a & b).

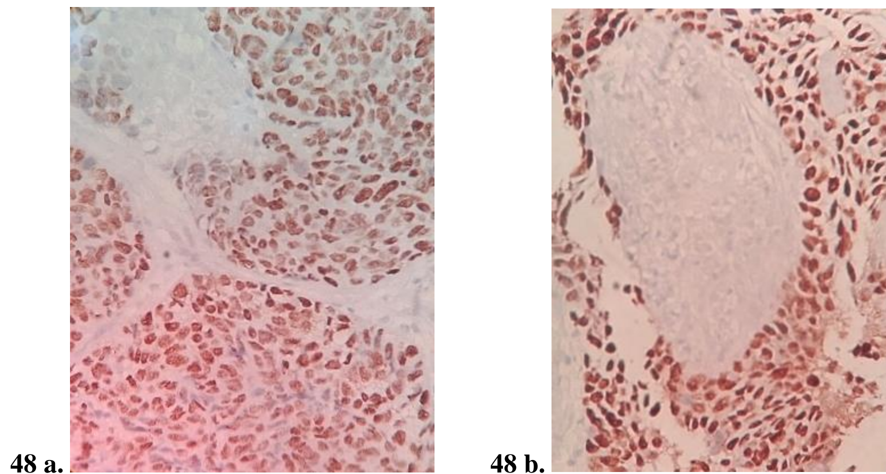



**Fig. 2 a&b** IHC positive nuclear staining for P53.

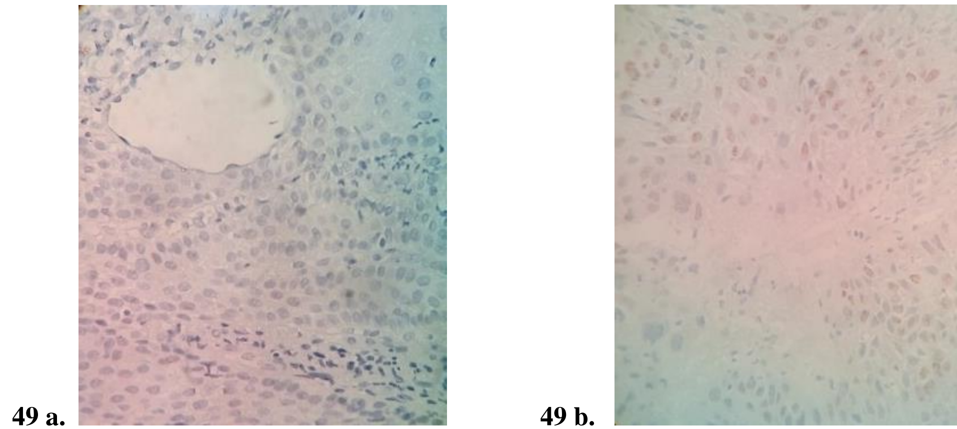



**Fig. 3 a&b** IHC negative nuclear staining for P53.

Table (3) shows TP53 affected exons and P53 protein accumulation in EC. It can be seen from the table that exon 5 is the most mutated exon with P53 accumulation in 5 cases unlike the other exons which show no protein accumulation at all, also 9 non-mutated samples revealed protein accumulation.

**Table 3.** Distribution of *TP53* mutation and p53 Protein Accumulation among different exons.

| Affected exon | p53 mutation 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 4.** *TP53* Mutation and p53 Accumulation with Clinicopathologic Correlations

| | | Age group | | Gender | | Histology | | |
|---|---|---|---|---|---|---|---|---|
| | | >56 | <56 | Male | Female | SCC | AC | UCA |
| **IHC accumulation** | **No** | 23 | 13 | 14 | 22 | 27 | 6 | 3 |
| | **Yes** | 8 | 6 | 3 | 11 | 13 | 1 | 0 |
| **TP53 mutation** | **No** | 19 | 11 | 9 | 21 | 25 | 3 | 2 |
| | **Yes** | 12 | 8 | 8 | 12 | 15 | 4 | 1 |

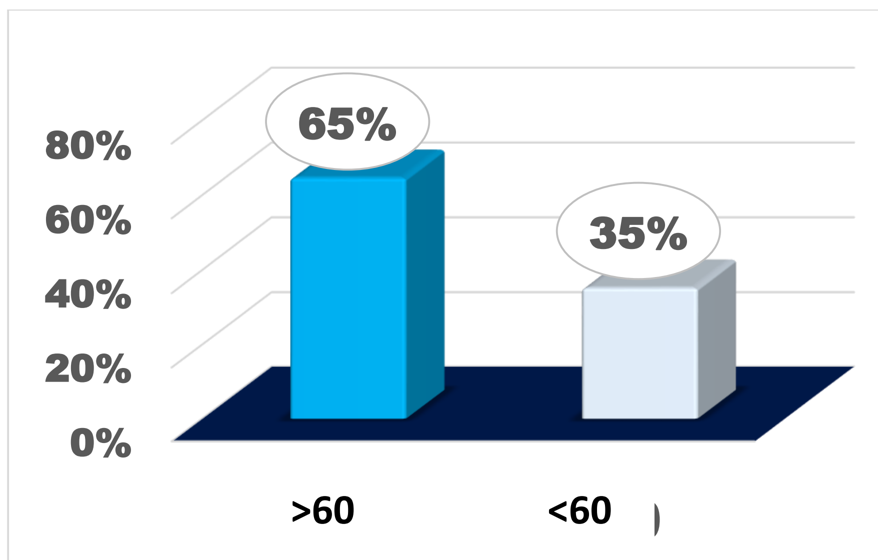



**Fig. 4** Age groups frequency.

**Table 5.** P53 protein accumulation and p53 gene mutation in esophageal cancer patients.

| Histology | N | M | IHC (-ve) | IHC (+ve) |
|---|---|---|---|---|
| **SCC** | 25 | 15 | 27 | 13 |
| **AC** | 3 | 4 | 6 | 1 |
| **UCA** | 2 | 1 | 3 | 0 |

N: normal, M: mutant.

## 4.3. Mutational analysis result:

The study revealed p53 gene mutations in 20 (40%) out of 50 specimens of esophageal carcinomas. Ten of these mutations appeared in patients samples were caused by single-nucleotide substitutions, among them, six were missense mutations leading to amino acid change, and the other four were synonymous mutations without any amino acid change and no frameshift mutation was detected. Six out of ten (60 %) p53 gene mutations occurred in exon 5 at codons 160, 161 (twice), 163, 164 and 175, with 2 SNPs occurring in codon 287 in the same sample. Two mutations were found in exon 6 at codon 215 and 222. Exon 7 was found to be free from any variations whereas two mutations were present in exon 8 at codon 298 and 305 (Fig. 52).

The results expose that only SNP in position E298V and E294D were predicted to be neutral polymorphism. All other SNP K292K, K291M, E287D, E287K, S215N, P153P, Y163Y, M160V, A161D, K164E, P152R were predicted to be disease related according to MutationTaster software.

16 out of 43 (37.2%) SSC samples were found to be mutated, 81.3% of them existed in exon 5 and 25% in exon 6, whereas adenocarcinoma exhibited a higher rate of mutations (57.1%) with 100%, 50% and 25% involvement of exon 5, 6 and 8 respectively.

Deleterious nsSNPs rates predicted by SIFT were the higher (88.8%) followed by I-Mutant suite (77.8%). PolyPhen results revealed semi-equal results for both tolerated and deleterious nsSNPs 44.5% and 55.5% respectively.

PhD-SNP report defines 7 of coding region SNPs as disease related polymorphism (Table, 12), while MutationTaster classifies all detected

SNPs as disease causing except E298V and E294D which were classified as neutral polymorphism (Table, 13).

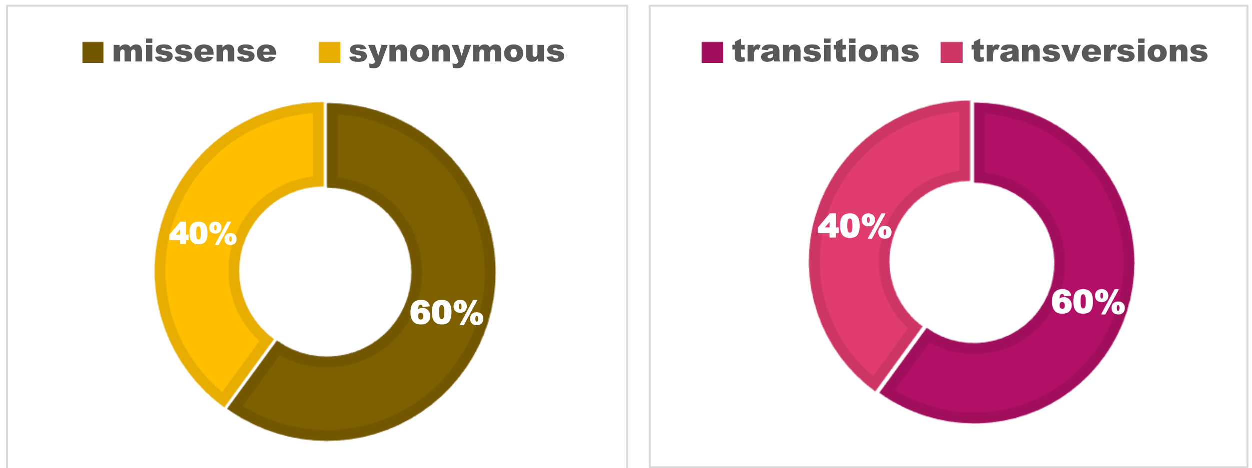



**Fig. 5** Distribution of different mutation types.

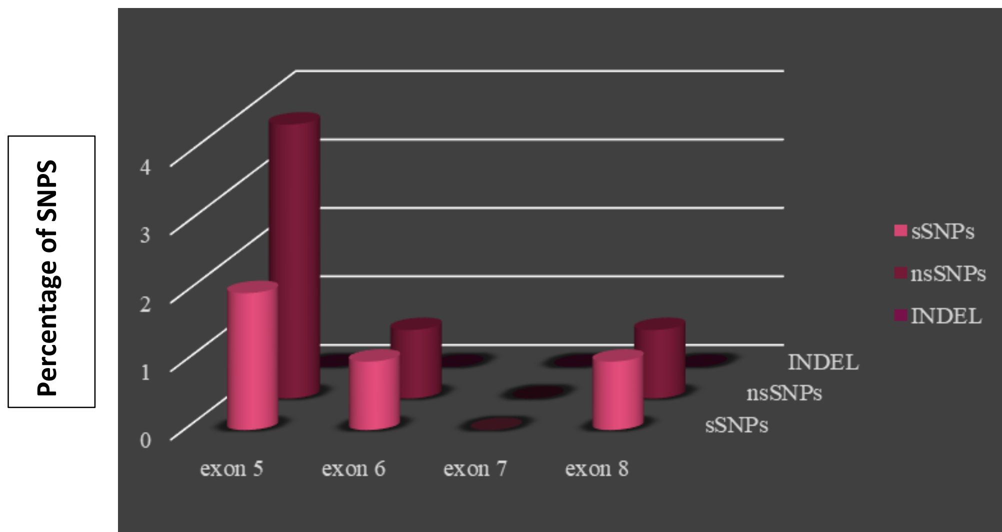



**Fig. 6** Distribution of TP53 coding synonymous SNPs (sSNPs), coding nonsynonymous SNPs (nsSNPs) and INDEL through exon 5-8 in P53 gene.

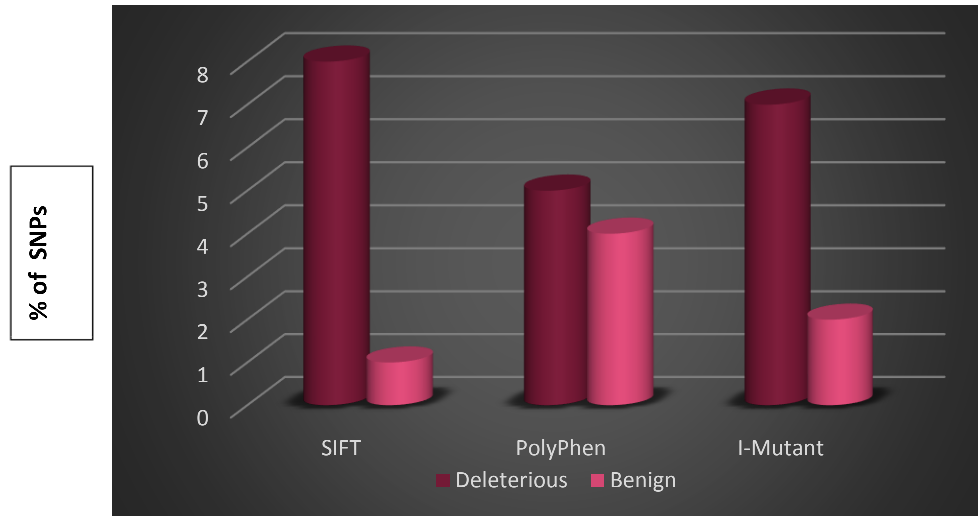



**Fig. 7 Distribution of deleterious and benign nsSNPs by SIFT, PolyPhen, and I-Mutant Suite.** The magenta cylindrical bar indicates the percentage of nsSNPs that were found to be deleterious by SIFT, damaging (Possibly/Probably) by PolyPhen, and largely unstable by I-Mutant Suite. The pink cylinder indicates the percentage of nsSNPs that were found to be tolerated by SIFT, benign by PolyPhen, and largely stable/neutral by I-Mutant Suite.

**Table 6:** The Prediction Results of nsSNPs of human *TP53* Using SIFT, PolyPhen and I-Mutant 3.0 algorithms.

| Prediction Result | SIFT | | PolyPhen | | I Mutant 3.0 | |
|---|---|---|---|---|---|---|
| | **No. of nsSNPs** | **%** | **No. of nsSNPs** | **%** | **No. of nsSNPs** | **%** |
| **Deleterious** | 4 | 66.7 % | 4 | 66.7 % | 4 | 66.7 % |
| **Tolerated** | 2 | 33.3 % | 2 | 33.3 % | 2 | 33.3 % |
| **Total** | 6 | 100% | 6 | 100% | 6 | 100% |

**Table 7:** List of variants that were analyzed using SIFT algorithm.

| Protein ID | Nucleotide change | AA Substitution | Prediction | Score | Median Info |
|---|---|---|---|---|---|
| **ENSP00000269305** | A/G | M160V | tolerant | 0.27 | 2.76 |
| **ENSP00000269305** | C/A | A161D | damaging | 0.01 | 2.76 |
| **ENSP00000269305** | A/G | K164E | damaging | 0.00 | 2.76 |
| **ENSP00000269305** | G/C | R175P | damaging | 0.00 | 2.76 |
| **ENSP00000269305** | G/A | S215N | damaging | 0.00 | 2.76 |
| **ENSP00000269305** | G/C | E298Q | tolerant | 0.39 | 2.75 |

**SIFT: Sorting Intolerant From Tolerant. SIFT score ≤ 0.05 considered damaging.**

**SIFT Tolerance Index: Ranges from 0 to 1.**

**Table 8**: Prediction of functionally significant nsSNPs by PolyPhen 2.0 algorithm.

| COSMIC ID | Amino acid change | Prediction | Score |
|---|---|---|---|
| **COSM44328** | M160V | Benign | **0.177** |
| **COSM44391** | A161D | Probably damaging | **1.000** |
| **COSM10762** | K164E | Probably damaging | **0.997** |
| **COSM45416** | R175P | Probably damaging | **1.000** |
| **COSM44093** | S215N | Probably damaging | **1.000** |
| **COSM45938** | E298Q | Benign | **0.003** |

**nsSNPs: Non-synonymous single nucleotide polymorphisms, PolyPhen: Polymorphism Phenotyping v2.**

**Table 9:** Prediction of nsSNPs stability status by I Mutant 2.0 algorithm.

| SNP ID'S | Amino acid change | SVM3 Prediction Effect | RI | ΔΔG (kcal/mol) |
|---|---|---|---|---|
| **COSM44328** | M160V | Large Decrease | 5 | -0.60 |
| **COSM44391** | A161D | Large Decrease | 3 | -0.52 |
| **COSM10762** | K164E | Neutral | 3 | -0.08 |
| **COSM45416** | R175P | Large Decrease | 0 | -0.57 |
| **COSM44093** | S215N | Large Decrease | 3 | 0.58 |
| **COSM45938** | E298Q | Neutral | 1 | -0.25 |

**I-mutant RI (Reliability Index): 0–10, where 0 is the lowest reliability and 10 is the highest reliability.**

**Table 10.** SNPs information and frequency among different samples.

| Alleles | Position in chromosome | Exon | AA change | Samples with the same SNP | SNP frequency Percentage |
|---|---|---|---|---|---|
| **A/G** | 7675134 | 5 | M160V | 9 out of 50 | 18 % |
| **C/A** | 7675130 | 5 | A161D | 1 out of 50 | 2 % |
| **A/G** | 7675122 | 5 | K164E | 14 out of 50 | 28 % |
| **G/C** | 7675088 | 5 | R175P | 1 out of 50 | 2 % |
| **G/A** | 7674887 | 6 | S215N | 1 out of 50 | 2 % |
| **G/C** | 7673728 | 8 | E298Q | 1 out of 50 | 2 % |

**Table 11.** Prediction of functionally significant nsSNPs, by PhD-SNP.

| Mutation | PhD-SNP | |
|---|---|---|
| | Prediction | RI |
| **K164E** | Disease-related polymorphism | 7 |
| **A161D** | Disease-related polymorphism | 9 |
| **M160V** | Disease-related polymorphism | 6 |
| **S215N** | Disease-related polymorphism | 5 |
| **E298Q** | Neutral | 5 |
| **R175P** | Disease-related polymorphism | 8 |

**Table 12.** Summary of SNP Characteristics identified in *TP53*gene exon 5, 6 and 8.

| Localization | Patient affected | alleles | AA Change | Position | SNP ID | Type | Significance* |
|---|---|---|---|---|---|---|---|
| **Exon 5** | 14 | A/G | K164E | 7675122 | rs879254249 | Missense | Disease causing |
| **Exon 5** | 1 | C/A | A161D | 7675130 | rs1064795691 | Missense | Disease causing |
| **Exon 5** | 9 | A/G | M160V | 7675134 | rs377274728 | Missense | Disease causing |
| **Exon 5** | 2 | C/T | Y163Y | 7675123 | COSM44391 | Coding-synonymous | Disease causing |
| **Exon 5** | 1 | G/C | R175P | 7675088 | COSM45416 | Missense | Disease causing |
| **Exon 5** | 1 | C/T | A161A | 7675129 | COSM44119 | Coding-synonymous | Disease causing |
| **Exon 6** | 1 | G/A | S215N | 7674887 | rs587782177 | Missense | Disease causing |
| **Exon 6** | 1 | G/C | P222P | 7674865 | COSM43924 | Coding-synonymous | Disease causing |
| **Exon 8** | 1 | A/T | E298Q | 7673728 | Novel | Missense | Polymorphism |
| **Exon 8** | 1 | G/A | K305K | 7673705 | COSM46382 | Coding-synonymous | Disease causing |

*according to MutationTaster.

**Table 13.** FATHMM server; cancer association predictions result of the non-synonymous changes found in *TP53* gene exon 5-8.

| COSMIC ID | Amino acid change | Prediction | Score |
|---|---|---|---|
| **COSM44328** | M160V | CANCER | **-9.03** |
| **COSM44391** | A161D | CANCER | **-9.34** |
| **COSM10762** | K164E | CANCER | **-9.12** |
| **COSM45416** | R175P | CANCER | **-9.93** |
| **COSM44093** | S215N | CANCER | **-9.58** |
| **COSM45938** | E298Q | CANCER | **-8.11** |

Regarding the Alanine to Aspartic Acid SNP at position 161 (Fig. 56), the mutant residue is bigger than the wild-type residue. The wild-type residue charge was neutral and the mutant residue charge is negative. The wild-type residue is more hydrophobic than the mutant residue.
The mutation is located within a stretch of residues annotated in UniProt as a special region: Interaction with CCAR2. The differences in amino acid properties can disturb this region and its function, and this point is shared between the six nsSNPs discussed here.

The wild-type and other residues have also been observed here. But neither the mutant residue nor another residue type with similar properties was observed at this position in other homologous sequences. So the mutation might be damaging to the protein.

The mutated residue is located in a domain that is important for the activity of the protein and in contact with another domain that is known to be involved in TP53 binding. The interaction between these domains could be disturbed by the mutation, which might affect the signal transduction between them and the function of the protein.

The mutated residue is located in contact with residues in a regulatory domain. It is possible that the mutation disturbs this interaction and thereby affects regulation of the catalalytic activity, and this point also were shared between all nsSNP discussed here except for SNP of Glutamic acid into Glutamine in position 298.There is a difference in charge between the wild-type and mutant amino acid in this SNP and all nsSNP except the SNP of Glutamic acid into Glutamine.

The mutant residue introduces a charge in a buried residue which can lead to protein folding problems. The wild-type and mutant amino acids differ in size. The mutant residue is bigger than the wild-type residue. The wild-type residue was buried in the core of the protein. The mutant residue is bigger and probably will not fit. The hydrophobicity of the wild-type and mutant residue differs. The mutation will cause loss of hydrophobic interactions in the core of the protein.

The second SNP of Serine into Asparagine at position 215 (Fig. 57). The mutant residue is bigger than the wild-type residue. The wild-type residue is more hydrophobic than the mutant one. The wild-type residue forms a hydrogen bond with Arginine at position 158. The size difference between wild-type and mutant residue makes the new residue not in the correct position to make the same hydrogen bond as the original wild-type residue does. Also the difference in hydrophobicity will affect hydrogen bond formation.

On the conservation level the wild-type residue is highly conserved, but a few other residue types have been observed at this position too. The mutant residue was among the residues at this position observed in other sequences. This means that homologous proteins exist with the same residue type as the mutant at this position and this mutation is possibly not damaging to the protein. The mutant residue is located near a highly conserved position.

Regarding amino acid properties wild-type and mutant amino acids differ in size. The mutant residue is bigger than the wild-type residue. The wild-type residue was buried in the core of the protein. The mutant residue is bigger and probably will not fit. The hydrophobicity of the wild-type and mutant

residue differs. The mutation will cause loss of hydrophobic interactions in the core of the protein.

Concerning the mutation of Arginine into Proline at position 175 (Fig. 58). The mutant residue is smaller than the wild-type residue. The wild-type residue charge is positive, while the mutant residue charge is neutral. The mutant residue is smaller and more hydrophobic than the wild-type residue. In the 3D-structure can be seen that the wild-type was involved in a metal-ion contact.

The size differences between the wild-type and mutant residue disturb the interaction with the metal-ion: "ZN". The difference in charge between wildtype (positive) and mutant residues (neutral) might also disturb the interaction with metal "ZN" which may lead to destabilization of the domain. The wild-type residue forms a hydrogen bond with: Methionine at position 237, Aspartic Acid at position 184, Proline at position 191, Aspartic Acid at position 184 and Methionine at position 237.

The size difference between wild-type and mutant residue makes the new residue is not in the correct position to make the same hydrogen bond as the original wild-type residue did. The difference in hydrophobicity will affect hydrogen bond formation. The wild-type residue forms a salt bridge with: Aspartic Acid at position 184. The difference in charge will disturb the ionic interaction made by the original, wild-type residue. Speaking about conservation level the wild-type residue is highly conserved, but a few other residue types have been observed at this position too.

Neither the mutant residue nor another residue type with similar properties was observed at this position in other homologous sequences. Based on conservation scores this mutation is probably damaging to the protein. The mutant residue is located near a highly conserved position.

There is a difference in charge between the wild-type and mutant amino acid. The charge of the buried wild-type residue is lost by this mutation. The wild-type and mutant amino acids differ in size. The mutant residue is smaller than the wild-type residue. The mutation will cause an empty space in the core of the protein. The hydrophobicity of the wild-type and mutant residue differs. The mutation will cause loss of hydrogen bonds in the core of the protein and as a result disturb correct folding.

In the case of alteration of Lysine into Glutamic Acid at position 164 (Fig. 59). The mutant residue is smaller than the wild-type residue. The wild-type residue charge was positive, the mutant residue charge is negative. The wild-type residue forms a hydrogen bond with: Glutamic Acid at position 271.

The size difference between wild-type and mutant residue makes the new residue not in the correct position to make the same hydrogen bond as the original wild-type residue does. The wild-type residue forms a salt bridge with: Glutamic Acid at position 271 and Glutamic Acid at position 285. The difference in charge will disturb the ionic interaction made by the original, wild-type residue.

The wild-type residue is very conserved, but a few other residue types have been observed at this position too. The mutant residue was not among the other residue types observed at this position in other homologous proteins.

However, residues that have some properties in common with the mutated residue were observed. This means that in some rare cases mutation might occur without damaging the protein.

There is a difference in charge between the wild-type and mutant amino acid. The mutation introduces the opposite charge at this position. This possibly disrupts contacts with other molecules. The wild-type and mutant amino acids differ in size being smaller in the mutant residue. This will cause a possible loss of external interactions.

About the SNP of Methionine into Valine at position 160 (Fig. 60). The mutant residue is smaller than the wild-type residue. The wild-type residue occurs often at this position in the sequence, but other residues have also been observed here. The mutant residue is among the other residue types that have been observed at this position in homologous sequences. This means that this mutation can occur at this position and is probably not damaging to the protein. The mutant residue is located near a highly conserved position.

The wild-type and mutant amino acids differ in size. The mutant residue is smaller than the wild-type residue. This will cause a possible loss of external interactions.

Finally SNP of a Glutamic Acid into a Glutamine at position 298 (Fig. 61) showed that wild-type residue charge is negative while the mutant residue charge is neutral.

There is a difference in charge between the wild-type and mutant amino acid. The charge of the wild-type residue will be lost, this can cause loss of interactions with other molecules or residues (Venselaar *et al.* 2010).

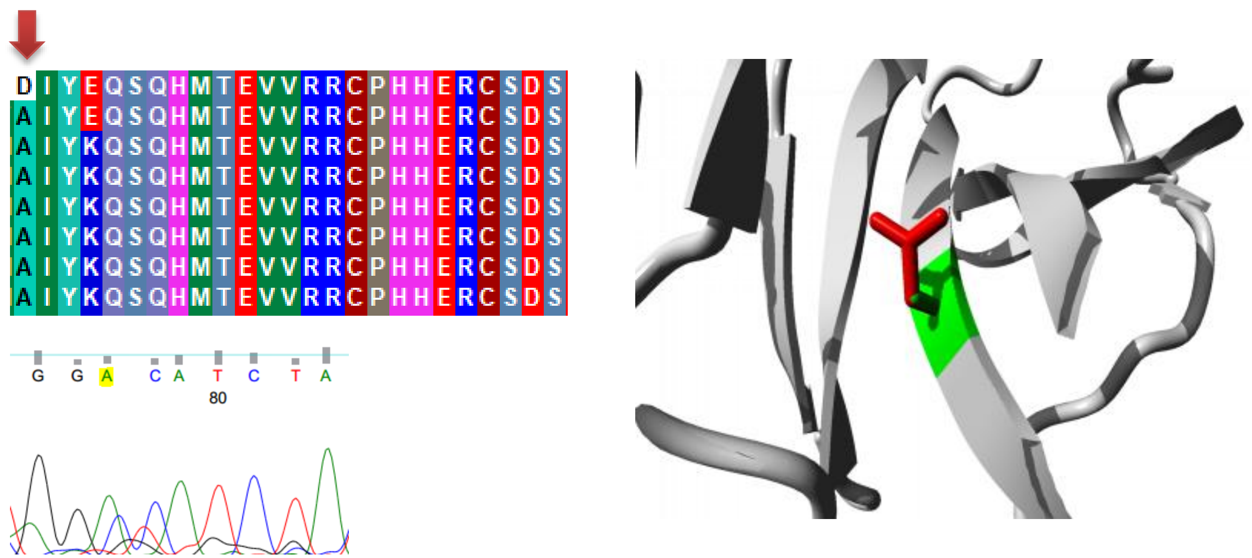



**Fig. 8** Mutation of alanine into aspartic acid at position 161.

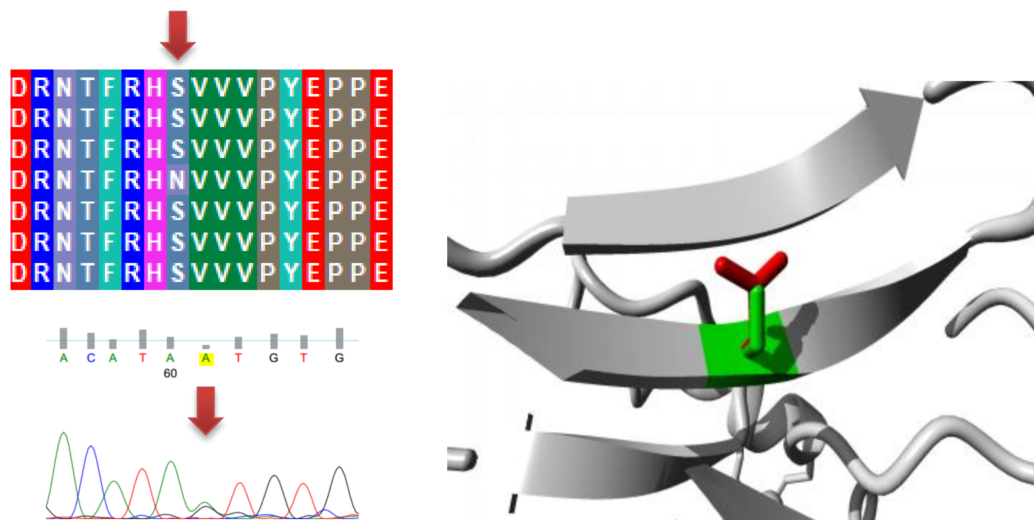



**Fig. 9** Mutation of a serine into asparagine at position 215.

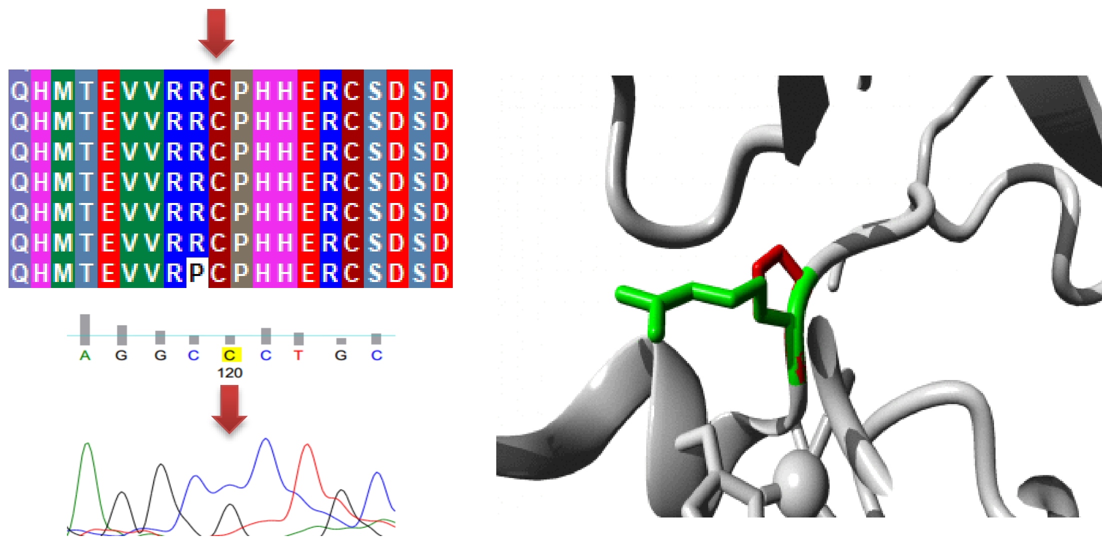



**Fig. 10** Mutation of arginine into proline at position 175.

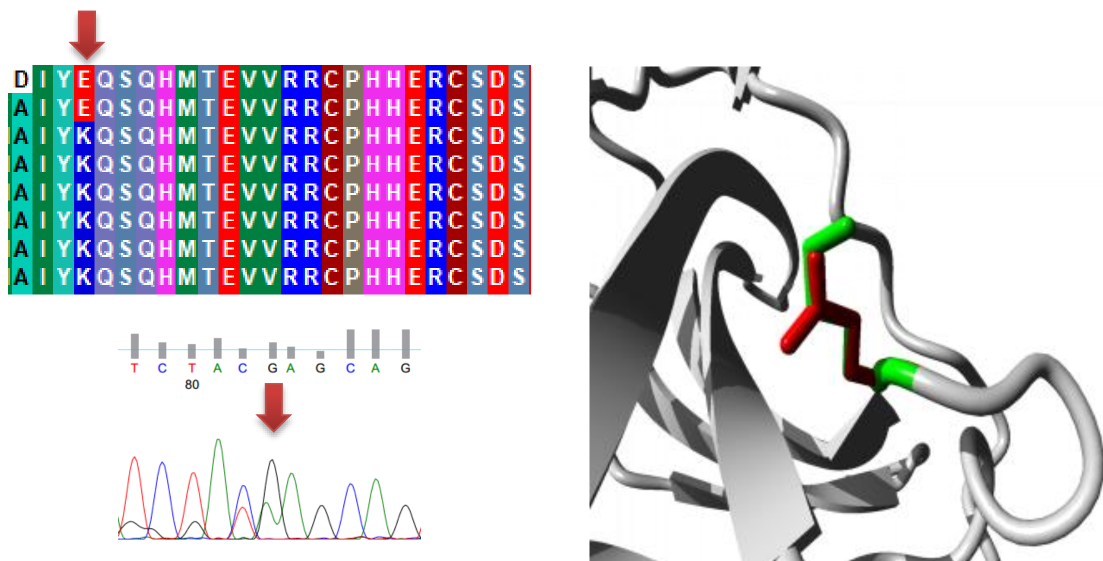



**Fig. 11** Mutation of lysine into glutamic acid at position 164.

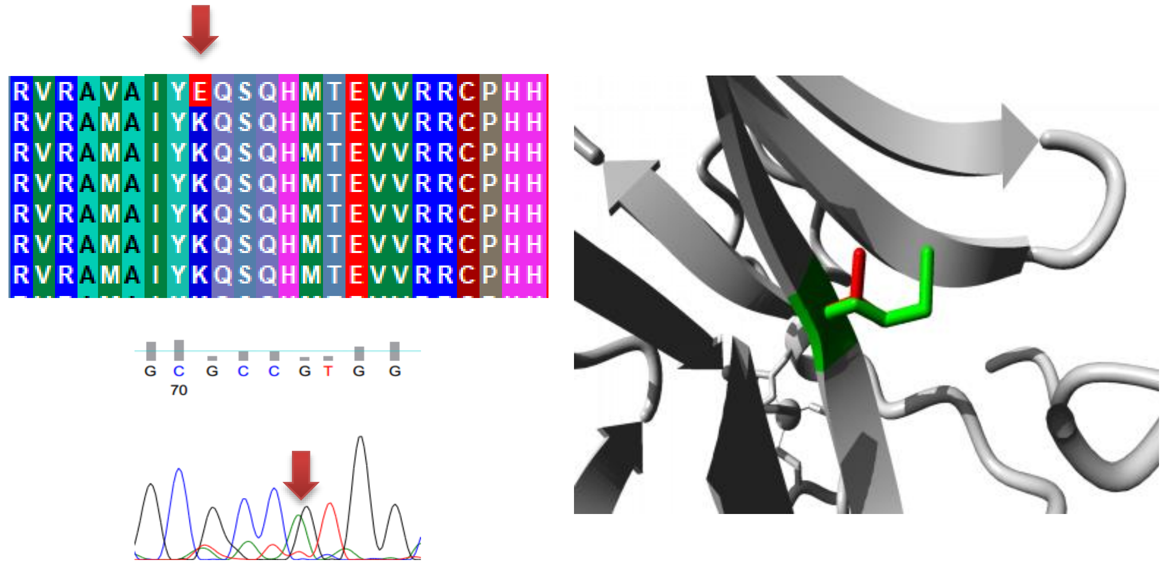



**Fig. 12** Mutation of a methionine into a valine at position 160.

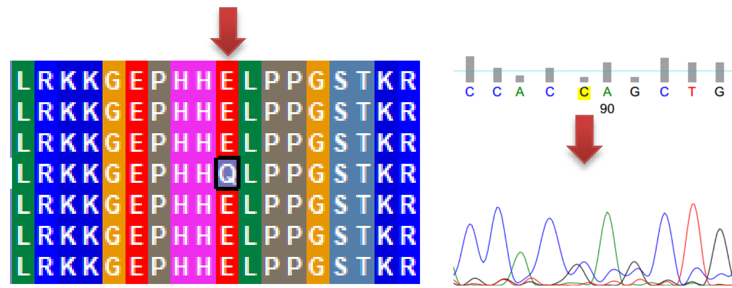



**Fig. 13** Mutation of glutamic acid into glutamine at position 298.

## 4.4. Statistical analysis:

### 4.4.1. H&E and IHC sample (No.= 204):

**Table 22.** Age group, sex, IHC result and histopathologic diagnosis frequencies of 204 EC samples.

| Age Group | Freq. & %* | Sex | Freq. & % | IHC Result | Freq. & % | H. Diagnosis * | Freq. & % |
|---|---|---|---|---|---|---|---|
| **<56** | 71 (34.8 %) | Male | 76 (37.3%) | +ve | 59 (27.6 %) | SCC | 169 (82.8%) |
| **>56** | 133 (65.2%) | Female | 128 (62.7%) | -ve | 155 (72.4%) | AC | 21 (10.3%) |
| | | | | | | UDC | 14 (6.9%) |
| **Total** | 204 (100%) | Total | 204 (100%) | Total | 204 (100%) | Total | 204 (100%) |

**Table 23.** Immunohistochemistry Result and Histopathologic Diagnosis Crosstab and Chi-square test.

| | | | Histopathologic Diagnosis | | | Total |
|---|---|---|---|---|---|---|
| | | | SCC | AC | UDC | |
| Immunohistochemistry Result | positive | Count | 53 | 2 | 1 | 56 |
| | | % of Total | 26.0% | 1.0% | 0.5% | 27.5% |
| | negative | Count | 116 | 19 | 13 | 148 |
| | | % of Total | 56.9% | 9.3% | 6.4% | 72.5% |
| Total | | Count | 169 | 21 | 14 | 204 |
| | | % of Total | 82.8% | 10.3% | 6.9% | 100.0% |

Pearson Chi-Square Value = 7.585a Sig. 0.023

**Table 24.** Age group and Histopathologic Diagnosis Crosstab and Chi-square test.

**age_groups * H. Diagnosis Crosstabulation**

Count

| | | H. Diagnosis | | | Total |
|---|---|---|---|---|---|
| | | SCC | ADENO | UDCA | |
| age_groups | <56 | 60 | 7 | 4 | 71 |
| | >56 | 109 | 14 | 10 | 133 |
| Total | | 169 | 21 | 14 | 204 |

Pearson Chi-Square value = 0.296a sig. 0. 862

**Table 25.** Relation between Sex and Histopathologic Diagnosis.

| | | | Histopathologic Diagnosis | | | Total |
|---|---|---|---|---|---|---|
| | | | SCC | AC | UDC | |
| Sex | F | Count | 117 | 7 | 4 | 128 |
| | | % of Total | 57.4% | 3.4% | 2.0% | 62.7% |
| | M | Count | 52 | 14 | 10 | 76 |
| | | % of Total | 25.5% | 6.9% | 4.9% | 37.3% |
| | Total | Count | 169 | 21 | 14 | 204 |
| | | % of Total | 82.8% | 10.3% | 6.9% | 100.0% |

Pearson Chi-Square value = 17.807a sig. 0.000.

From the above table the relation between Sex and Histopathologic Diagnosis were found to be significant, with SCC being more prevalent in females, AC and UDC in males.

## 4.4.2. H&E, IHC and mutation analysis of samples (No.= 50):

**Table 14.** Age group, sex, mutational status, IHC result and histopathologic diagnosis frequencies.

| Age Group | Freq. & %* | Sex | Freq. & % | M. Status* | Freq. & % | IHC Result | Freq. & % | H. Diagnosis * | Freq. & % |
|---|---|---|---|---|---|---|---|---|---|
| <56 | 19 (38%) | Male | 17 (34%) | mutated | 20 (40%) | +ve | 14 (28%) | SCC | 40(80%) |
| >56 | 31 (62%) | Female | 33 (66%) | normal | 30 (60%) | -ve | 36 (72%) | AC | 7 (14%) |
| | | | | | | | | UCA | 3(6%) |
| Total | 50 (100%) | Total | 50 (100%) | Total | 50 (100%) | Total | 50 (100%) | Total | 50 (100%) |

Freq. & %* = frequencies and percentages

M. Status* = mutational status

H. Diagnosis* = histopathologic diagnosis

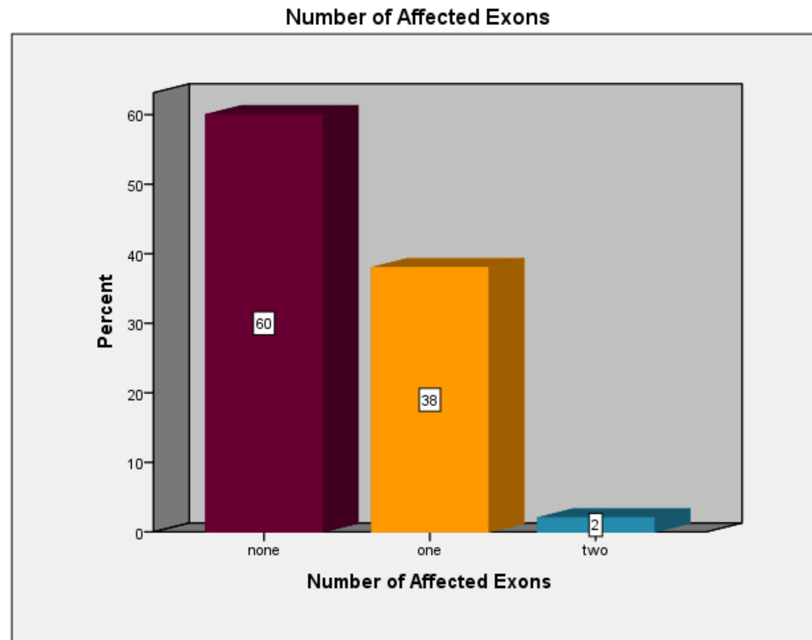



**Fig 14.** Number of Affected Exons frequency.

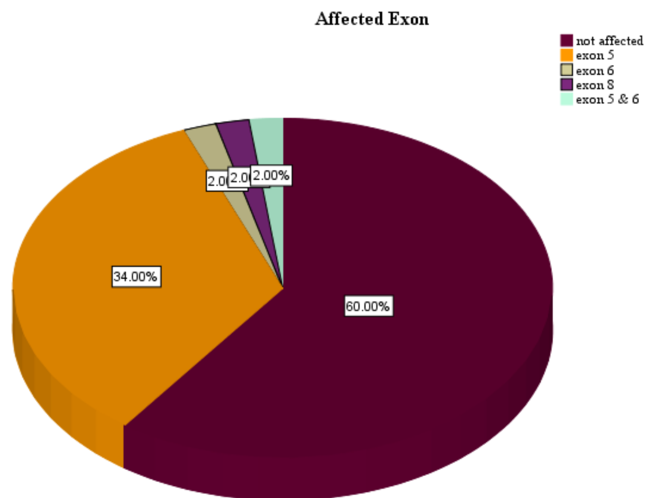



**Fig 15.** Affected Exons frequency.

**Table 15.** Relationship between histopathologic diagnosis and mutational status.

| | | | Mutational Status | | Total |
|---|---|---|---|---|---|
| | | | yes | no | |
| H. Diagnosis | SCC | Count | 15 | 25 | 40 |
| | | % of Total | 30.0% | 50.0% | 80.0% |
| | AC | Count | 4 | 3 | 7 |
| | | % of Total | 8.0% | 6.0% | 14.0% |
| | UCA | Count | 1 | 2 | 3 |
| | | % of Total | 2.0% | 4.0% | 6.0% |
| Total | | Count | 20 | 30 | 50 |
| | | % of Total | 40.0% | 60.0% | 100.0% |

Pearson Chi-Square value = 1.017a Sig. 0.601

**Table 16.** Relationship between Histopathologic Diagnosis and Number of Affected Exons.

| | | | Number of Affected Exons | | | Total |
|---|---|---|---|---|---|---|
| | | | none | one | Two | |
| H. Diagnosis | SCC | Count | 25 | 14 | 1 | 43 |
| | | % of Total | 50.0% | 28.0% | 2.0% | 86.0% |
| | AC | Count | 3 | 4 | 0 | 7 |
| | | % of Total | 6.0% | 8.0% | 0.0% | 14.0% |
| | UDC | Count | 2 | 1 | 0 | 3 |
| | | % of Total | 4.0% | 2.0% | 0.0% | 6.0% |
| Total | | Count | 30 | 19 | 1 | 50 |
| | | % of Total | 60.0% | 38.0% | 2.0% | 100.0% |

Pearson Chi-Square value = 1.444a Sig. 0.837

**Table 17.** Relationship between Histopathologic Diagnosis and Affected Exons.

| | | | Affected Exon | | | | | Total |
|---|---|---|---|---|---|---|---|---|
| | | | not affected | exon 5 | exon 6 | exon 8 | exon 5 & 6 | |
| H. Diagnosis | SCC | Count | 25 | 12 | 1 | 1 | 1 | 40 |
| | | % of Total | 50.0% | 24.0% | 2.0% | 2.0% | 2.0% | 80.0% |
| | AC | Count | 3 | 4 | 0 | 0 | 0 | 7 |
| | | % of Total | 6.0% | 8.0% | 0.0% | 0.0% | 0.0% | 14.0% |
| | UDC | Count | 2 | 1 | 0 | 0 | 0 | 3 |
| | | % of Total | 4.0% | 2.0% | 0.0% | 0.0% | 0.0% | 6.0% |
| Total | | Count | 30 | 17 | 1 | 1 | 1 | 50 |
| | | % of Total | 60.0% | 34.0% | 2.0% | 2.0% | 2.0% | 100.0% |

Pearson Chi-Square value = 2.448a Sig. 0.964

**Table 18.** Relationship between histopathologic diagnosis and immunohistochemistry result.

| | | | Immunohistochemistry Result | | Total |
|---|---|---|---|---|---|
| | | | positive | Negative | |
| H. Diagnosis | SCC | Count | 13 | 27 | 40 |
| | | % of Total | 26.0% | 54.0% | 80.0% |
| | AC | Count | 1 | 6 | 7 |
| | | % of Total | 2.0% | 12.0% | 14.0% |
| | UDC | Count | 0 | 3 | 3 |
| | | % of Total | 0.0% | 6.0% | 6.0% |
| Total | | Count | 14 | 36 | 50 |
| | | % of Total | 28.0% | 72.0% | 100.0% |

Pearson Chi-Square value = 2.222a sig. 0.329

The association between TP53 mutation and P53 accumulation is presented in table 19. No association were found between them.

**Table. 19.** Relationship between immunohistochemistry result and mutational status.

| | | | Mutational Status | | Total |
|---|---|---|---|---|---|
| | | | yes | no | |
| IHC Result | positive | Count | 5 | 9 | 14 |
| | | % of Total | 10.0% | 18.0% | 28.0% |
| | negative | Count | 15 | 21 | 36 |
| | | % of Total | 30.0% | 42.0% | 72.0% |
| Total | | Count | 20 | 30 | 50 |
| | | % of Total | 40.0% | 60.0% | 100.0% |

Pearson Chi-Square = 0.149a sig. 0.700.

**Table 20.** Relationship between immunohistochemistry result and number of affected exons.

| **Crosstab** | | | | | | |
|---|---|---|---|---|---|---|
| | | | Number of Affected Exons | | | Total |
| | | | none | one | two | |
| IHC Result | positive | Count | 9 | 5 | 0 | 14 |
| | | % of Total | 18.0% | 10.0% | 0.0% | 28.0% |
| | negative | Count | 21 | 14 | 1 | 36 |
| | | % of Total | 42.0% | 28.0% | 2.0% | 72.0% |
| Total | | Count | 30 | 19 | 1 | 50 |
| | | % of Total | 60.0% | 38.0% | 2.0% | 100.0% |

Pearson Chi-Square Value = 0.475a sig. 0.789

**Table 21.** Relationship between immunohistochemistry result and affected exon.

**Crosstab**

| | | | Affected Exon | | | | | Total |
|---|---|---|---|---|---|---|---|---|
| | | | not affected | exon 5 | exon 6 | exon 8 | exon 5 & 6 | |
| IHC Result | positive | Count | 9 | 5 | 0 | 0 | 0 | 14 |
| | | % of Total | 18.0% | 10.0% | 0.0% | 0.0% | 0.0% | 28.0% |
| | negative | Count | 21 | 12 | 1 | 1 | 1 | 36 |
| | | % of Total | 42.0% | 24.0% | 2.0% | 2.0% | 2.0% | 72.0% |
| Total | | Count | 30 | 17 | 1 | 1 | 1 | 50 |
| | | % of Total | 60.0% | 34.0% | 2.0% | 2.0% | 2.0% | 100.0% |

Pearson Chi-Square Value = 1.243a sig. 0.871

# CHAPTER FIVE

## DISCUSSION, CONCLUSION AND RECOMENDATIONS

# CHAPTER FIVE

# DISCUSSION CONCLUSION AND RECOMMENDATIONS

## 5.1 Discussion

Esophageal cancer is a common and fatal cancer with great regional and ethnic variations in incidence and risk factors. The histopathological results of the EC biopsies examined here in this study. Revealed that 83.3% were SCC and 10.4% were AC, This is in line with previous reports indicating that SCC and AC are the main subtypes of human EC. Both tumors showed typical histological features for both subtypes as described in many reports (Gupta *et al.* 2017). TP53 gene mutation and p53 protein accumulation have been investigated in both subtyped diagnosed.

P53 protein in normal cells is a labile protein of low quantity, but cellular stress can generate an increase in its level to compensate its main function as a guardian of the genome (Oren 1999; Pflaum *et al.* 2014). Mutations of *TP53* gene can lead to loss of functional characteristics in tumor cells, as mutant *TP53* may not play the assigned role in repairing cellular machinery leading to a loss of normal function and, subsequently, cells with mutant gene may express uncontrolled replication which leads to accumulation of protein 53 (IARC [n.d.]).

In the present study the presence of TP53 gene mutation were investigated here in 50 Sudanese patients with EC. Patients mostly were females (62.7%) with SCC constituted 84% of the cases, the predominant subtype. Twenty

(40%) of the patients showed p53 gene mutation. The frequency of mutations observed was moderate in contrast to similar previous reports.

İmazeki *et al.* (1992) observed mutations in the TP53 gene in 80% of EC which is very high compared to our finding, this may be attributed to false effect of the small sample size (5 cases) they used in their study.

The reported frequencies of p53 gene mutation in EC varied widely from 17 to 84%, and there are several reasons for this variation. Including the variation in the methods used in mutation detection and the interpretation of the results. In this study we used the PCR direct sequencing technique, another factor of variation is the number of the screened exons and regulatory regions which vary between different studies.
*TP53* mutations were found in 22.9% of esophageal SCCs and 14.3% of esophageal ACs in the study reported by Zheng *et al.* (2016). Shi *et al*. (1999), in Henan Province, China, detected p53 gene mutation in 30/43 (70%) SCC cases. In another study in Henan Province Li-Ya *et al.* (2005) detected *TP53* gene mutations in 40.9% of their EC specimens. On the other hand Zheng *et al*. (2016) reported higher mutation rate in SCC samples compared to 31.4% for AC samples in Qiqihar city, China. In this study TP53 gene mutations were found as 37% of the SCC specimens and 57.1% for AC. As can be seen there are variations between the results obtained by various authors including the present findings. This could be due to several factors including sample size, type of exons studied and geographical zone.

Therefore, It seems difficult to compare the results of the current study previous reports because of the differences of antibodies used, antigen

retrieval methods and solutions used and interpretation criteria followed to evaluate the results (Coggi et al. 1997).

Exon 5 of the TP53 gene was observed to be the most mutated among the other exons investigated in this study; 17/20 (85%) of all mutations were detected in exon 5 compared to 2/20 (10%) in exon 6, 1/20 (5%) in exon 8, and no mutation in exon 7. In comparison, Uchino *et al.* (1996) reported mutation distribution of 39.3% in exon 5, 32.1% in exon 6, 17.9% in exon 7 and (10.7%) in exon 8. This partly agrees with our result in that exon 5 has the higher mutation rate, the differences in other exons can be attributed to difference in sample size between the two studies.

Ten percent of all detected mutations are classified as neutral polymorphism, while 90% mutations are considered as high rate disease-causing mutation according to MutationTaster. This rate would be less using other software due to different algorithms used, database linked and characteristics of the different software.

The highest incidence of EC in men has been noticed in China (21.6/100,000), Kazakhstan (19.1/100,000) and in part of African countries (12.5–19.2/100,000). In the USA, incidence of 2.1 and 1.2 per 100,000 in men and women has been reported (Liao *et al.* 2007). Internationally, SCC is more commonly diagnosed in blacks and white females, whereas AC is more common among white males (Baquet *et al.* 2005).
Male to female ratio noticed here differs from the global statistics, more females are affected with EC than males (62.7%) and (37.3%) respectively; and this is consistent with the statistics from the Radioisotope Center, Khartoum, for EC in 12 years (2000-2012) (RICK 2013) and a study done

by Hamad *et al.* (2017) and Mohammed *et al.* (2012). Specific local risk factors may exist predisposing females for EC more than males. Regarding TP53 gene mutations 8/20 (40%) males had mutations versus 12/20 (60%) females and this match well with the above statistics of female predominance among EC patients.

In this study *TP53* mutations in exons 5 - 8 were detected in 40% of the EC patients whereas p53 accumulation was observed in 28% only. This result is approximate to that of Uchino *et al.* (1996) who reported p53 mutation in 34% of EC cases. This study concludes that mutations in p53 gene are not always associated with *p53* protein accumulation, as discordant cases reached 48%. Coggi et al. 1997 found *TP53* mutations in exons 5 - 8 in 53% of the carcinomas whereas p53 accumulation was observed in 57% of the cases. Discordant cases represented 38%, whereas 27% of the cases did not display p53 mutation or accumulation.

Discordance of TP53 gene mutation and p53 protein accumulation, proposing that p53 function may be affected by mechanisms other than mutation. So P53 protein accumulation cannot be considered as indicator for genetic alteration in esophageal tumors.

Levine *et al.* (1991) stated that there are at least three mutation 'hotspots' affecting residues 175, 248, and 273 of p53 protein. The highest frequency of mutations (13%) had been found at 273 position. In this study only one sample was found to have mutation in 175 hotspot which represent 2% of the sample.

Hollstein *et al.* (1991) reviewed the list of mutations in the evolutionarily conserved codons of P53 in different types of human cancer. They found that transitions predominated in colon, brain, and lymphoid malignancies, whereas G:C to T:A transversions are the most frequent substitutions observed in lung and liver cancers. Mutations at A:T base pairs were more frequent in esophageal carcinomas than in other solid tumors and this disagrees with our finding; there was no mutation at A:T base pairs and transitions were most frequent in this study than transversions, 6:4 respectively.

Hollstein *et al.* (1990) studied 4 human esophageal carcinoma cell lines and 14 human esophageal SCC and identified a mutated p53 allele (1 frameshift and 6 missense mutations) in 2 cell lines and in 5 of the tumor specimens. All missense mutations occurred at G: C base pairs in codons at or adjacent to mutations previously reported in other cancers. In this study all missense mutations occurred at A: G (33.3%), G: C (33.3%), C:A (16.7%) and G:A (16.7%) base pairs while all silent mutations occurred at C:T (50%), G:C (25%) and G:A (25%).

No statistical significance or correlation was found between immunohistochemistry, mutational status, sex, age and histopathologic type, nor between mutational status and p53 accumulation. This seems to agree with previous finding indicating that clinicopathological factors have no association with p53 mutation (Shi *et al.* 1999) . However, Okuda et al. 2001 found that incidence of p53 gene mutation can be influenced by age, being higher in the young.

Molecular study of the TP53 gene can provide valuable essential data on the biology of cancer in general and that of EC in particular, and can also help in introducing a solid base for targeted gene therapy studies and genome wide association studies if linked to patients clinicopathological features, survival, and compared to control group. In conclusion TP53 gene mutation or protein accumulation can't be studied in a discrete way without considering the different main players affecting tumorigenesis. By doing this in big cancer projects more valuable data can be obtained and analyzed in order to complete the picture and provide more reliable and consistent information about EC.

# CONCLUSION AND RECOMMENDATIONS

## 5.2 Conclusion

On the base of this study, we concluded the following:

The most prevalent esophageal cancer in Sudanese patients is squamous cell carcinoma which represents a majority of EC cases.

Sudanese females were more affected with EC than males contrary to global epidemiologic data.

There is a significant relationship between sex and histopathologic diagnosis with SCC being more prevalent in females, AC and UDC in males.

The majority of esophageal cancer cases were found in population over 60 years old.

Positive P53 immunohistochemical results were approximately doubled in SCCs compared to AC cases.

Mutation of exon 5 in TP53 gene were the most frequent in EC. Genomic results have identified a high TP53 mutation rate in esophageal adenocarcinoma compared to SCC.

Most of nsSNPs detected in TP53 gene were found to be damaging depending on different bioinformatics analysis tools used in this study.

## 5.3. Recommendations

On the bases of this study we recommend:

Further studies using fresh tissue samples instead of FFPE samples are recommended to obtain abundant high quality DNA for genomic based studies and avoid short sequence problems.

Forthcoming studies of TP53 gene should scan the entire gene specially 3’ and 5’ UTR regions to deeply understand the relationship between the mutations of this gene and different pathogenesis mechanisms behind it.

National survey programs for screening of EC in populations above 60 years should be established due to asymptomatic nature of the disease in the first stages and its high mortality rate.

# REFERENCES


(MFMER), M.F. for M.E. and R. (2015). *Adjuvant Therapy: Treatment to Keep Cancer from Returning*. Available at: https://www.mayoclinic.org/diseases-conditions/cancer/in-depth/adjuvant-therapy/ART-20046687.

Adzhubei, I., Jordan, D.M. and Sunyaev, S.R. (2013). Predicting functional effect of human missense mutations using PolyPhen-2. *Current protocols in human genetics*:7–20.

Adzhubei, I.A., Schmidt, S., Peshkin, L., Ramensky, V.E., Gerasimova, A., Bork, P., … Sunyaev, S.R. (2010). A method and server for predicting damaging missense mutations. *Nature methods* **7**(4):248.

Al2sarraf, M., Martz, K. and Herskovic, A. (1997). Progress report of combined chemoradiotherapy versus radiotherapy alone in pa2 tients with esophageal cancer: an intergroup study. *J Clin Oncol* **15**(2):2772284.

Allen, W.E. (2008). diFiore's Atlas of Histology with Functional Correlations (11th Edition). *Journal of Anatomy* **213**(3):357–358. doi: https://doi.org/10.1111/j.1469-7580.2008.00956.x.

Aloia, T.A., Harpole, D.H., Reed, C.E., Allegra, C., Moore, M.-B.H., Herndon, J.E. and D'amico, T.A. (2001). Tumor marker expression is predictive of survival in patients with esophageal cancer. *The Annals of thoracic surgery* **72**(3):859–866.

Analytic Jena (2017). *Instructions for Use Life Science Kits & Assays InnuPREP PCRpure Kit*.

Araújo, C.M.M., Souhami, L., Gil, R.A., Carvalho, R., Garcia, J.A., Froimtchuk, M.J., … Canary, P.C. V (1991). A randomized trial comparing radiation therapy versus concomitant radiation therapy and chemotherapy in carcinoma of the thoracic esophagus. *Cancer* **67**(9):2258–2261.

Arnal, M.J.D., Arenas, Á.F. and Arbeloa, Á.L. (2015). Esophageal cancer: Risk factors, screening and endoscopic treatment in Western and Eastern countries. *World journal of gastroenterology: WJG* **21**(26):7933.

Badakhshi, H., Kaul, D. and Zhao, K. (2016). Association between the inflammatory biomarker, C-reactive protein, and the response to radiochemotherapy in patients with esophageal cancer. *Molecular and clinical oncology* **4**(4):643–647.

Badwe, R.A., Sharma, V., Bhansali, M.S., Dinshaw, K.A., Patil, P.K., Dalvi, N., … Desai, P.B. (1999). The quality of swallowing for patients with operable esophageal carcinoma. *Cancer* **85**(4):763–768.

Balmadrid, B. and Hwang, J.H. (2015). Endoscopic resection of gastric and esophageal cancer. *Gastroenterology report* **3**(4):330–338.

Bamford, S., Dawson, E., Forbes, S., Clements, J., Pettett, R., Dogan, A., … Stratton, M.R. (2004). The COSMIC (Catalogue of Somatic Mutations in Cancer) database and website. *British journal of cancer* **91**(2):355.

Baquet, C.R., Commiskey, P., Mack, K., Meltzer, S. and Mishra, S.I. (2005). Esophageal cancer epidemiology in blacks and whites: racial and gender disparities in incidence, mortality, survival rates and histology. *Journal of the National Medical Association* **97**(11):1471.

Betts, J.G., DeSaix, P., Johnson, E., Johnson, J.E., Korol, O., Kruse, D.H.,

… Young, K.A. (2014). Anatomy and physiology.

Bio-Rad *Sequencing and Bioinformatics Module Instruction Manual*. California, USA.

Boyle, J. (2014). Molecular Biology, David P. Clark and Nanette J. Pazdernik, Academic Press- Cell, 2012, 928 pp., ISBN 978- 0- 1237–8594- 7, $135.00 (hardback). *Biochemistry and Molecular Biology Education* **42**(1):100–101.

Bray, F., Ferlay, J., Soerjomataram, I., Siegel, R.L., Torre, L.A. and Jemal, A. (2018). Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA: a cancer journal for clinicians*.

Butler, J.M. (2011). *Advanced Topics in Forensic DNA Typing: Methodology*. Academic Press.

Cancer.Net (2017). *Difficulty Swallowing or Dysphagia*. Available at: https://www.cancer.net/navigating-cancer-care/side-effects/difficulty-swallowing-or-dysphagia.

Cao, W., Chen, X., Dai, H., Wang, H., Shen, B., Chu, D., … Zhang, Z.-F. (2004). Mutational spectra of p53 in geographically localized esophageal squamous cell carcinoma groups in China. *Cancer* **101**(4):834–844.

Capriotti, E., Altman, R.B. and Bromberg, Y. (2013). Collective judgment predicts disease-associated single nucleotide variants. *BMC genomics* **14**(3):S2.

Capriotti, E., Fariselli, P. and Casadio, R. (2005). I-Mutant2. 0: predicting

stability changes upon mutation from the protein sequence or structure. *Nucleic acids research* **33**(suppl_2):W306--W310.

Central Bureau of Statistics Sudan (2017). *Republic of the Sudan*. Available at: www.citypopulation.de/Sudan.html.

Chava, S., Mohan, V., Shetty, P.J., Manolla, M.L., Vaidya, S., Khan, I.A., … Hasan, Q. (2012). Immunohistochemical evaluation of p53, FHIT, and IGF2 gene expression in esophageal cancer. *Diseases of the Esophagus* **25**(1):81–87.

Chen, G., Wang, Z., Liu, X. and Liu, F. (2009). Adjuvant radiotherapy after modified Ivor-Lewis esophagectomy: can it prevent lymph node recurrence of the mid-thoracic esophageal carcinoma? *The Annals of thoracic surgery* **87**(6):1697–1702.

Chen, I. (2018). Immunotherapy in Cancer Treatment: A Review of Checkpoint Inhibitors. *US Pharm* **43**(2):27–31.

Chou, James C. Gress, F.G. (2011). *Benign Esophageal Tumors.health.am*. Available at: http://www.health.am/cr/benign-esophageal-tumors/.

Chung, C.-S., Lee, Y.-C. and Wu, M.-S. (2015). Prevention strategies for esophageal cancer: Perspectives of the East vs. West. *Best Practice & Research Clinical Gastroenterology* **29**(6):869–883.

Coggi, G., Bosari, S., Roncalli, M., Graziani, D., Bossi, P., Viale, G., … Blandamura, S. (1997a). p53 protein accumulation and p53 gene mutation in esophageal carcinoma. *Cancer* **79**(3):425–432.

Coggi, G., Bosari, S., Roncalli, M., Graziani, D., Bossi, P., Viale, G., …

Blandamura, S. (1997b). p53 protein accumulation and p53 gene mutation in esophageal carcinoma: a molecular and immunohistochemical study with clinicopathologic correlations. *Cancer: Interdisciplinary International Journal of the American Cancer Society* **79**(3):425–432.

Corwin, E.J. (2008). Handbook of Pathophysiology. 3rd ed. Lippincott Williams & Wilkins.

D'Antoni, A. V. (2016). *Gray's Anatomy. The Anatomical Basis of Clinical Practice.*

Dai, Y., Li, C., Xie, Y., Liu, X., Zhang, J., Zhou, J., … Yang, S. (2014). Interventions for dysphagia in oesophageal cancer ( Review ) Interventions for dysphagia in oesophageal cancer. (10):4–6. doi: https://doi.org/10.1002/14651858.CD005048.pub4.Copyright.

Dicato, M., Plawny, L. and Diederich, M. (2010). Anemia in cancer. *Annals of Oncology* **21**(suppl_7):vii167-vii172.

Doss, C.G.P., Rajith, B., Garwasis, N., Mathew, P.R., Raju, A.S., Apoorva, K., … Dike, I.P. (2012). Screening of mutations affecting protein stability and dynamics of FGFR1-A simulation analysis. *Applied and Translational Genomics* **1**:37–43. doi: https://doi.org/10.1016/j.atg.2012.06.002.

Ek, W.E., Levine, D.M., D'Amato, M., Pedersen, N.L., Magnusson, P.K.E., Bresso, F., … MacGregor, S. (2013). Germline genetic contributions to risk for esophageal adenocarcinoma, barrett's esophagus, and gastroesophageal reflux. *Journal of the National Cancer Institute* **105**(22):1711–1718. doi: https://doi.org/10.1093/jnci/djt303.

Ellis, H. and Mahadevan, V. (2013). *Clinical Anatomy Applied Anatomy for*

*Students and Junior Doctors*. 13th ed. John Wiley & Sons, Ltd Registered.

Eloubeidi, M.A., Desmond, R., Arguedas, M.R., Reed, C.E. and Wilcox, C.M. (2002). Prognostic Factors for the Survival of Patients with Esophageal Carcinoma in the U.S. *American cancer society* **95**(5):1434–1443. doi: https://doi.org/10.1002/cncr.10808.

Fisher, O.M., Lord, S.J., Falkenback, D., Clemons, N.J., Eslick, G.D. and Lord, R. V (2016). The prognostic value of TP53 mutations in oesophageal adenocarcinoma: a systematic review and meta-analysis. *Gut*:gutjnl-2015.

Freed-Pastor, W.A. and Prives, C. (2012). Mutant p53: One name, many proteins. *Genes and Development* **26**(12):1268–1286. doi: https://doi.org/10.1101/gad.190678.112.

Geohive (2008). *No Title*. Available at: https://web.archive.org/web/20170201111708/http:/geohive.com/cntry/sudan.aspx.

Goljan, E.F. (2007). *Rapid Review Pathology*. second. Elsevier Inc.

Gray, H. and Standring, S. (2008). Gray's anatomy: the anatomical basis of clinical practice, 40th edn. Churchill-Livingstone.

Gupta, V., Bhardwaj, S. and Bhagat, O.K. (2017). Pattern of esophageal cancer in tertiary care hospital in North India: a clinicopathological study. *International Journal of Research in Medical Sciences* **5**(4):1405–1409.

Hamad, A.M., Ahmed, M.E.K., Abdelgadir, A. and Suliman, I.B. (2017). Esophageal Cancer in Sudan: Demographic and Histopathlogical Variations. *Open Journal of Gastroenterology* **7**(03):124.

Hardikar, S., Onstad, L., Song, X., Wilson, A.M., Montine, T.J., Kratz, M., … White, E. (2014). Inflammation and oxidative stress markers and esophageal adenocarcinoma incidence in a Barrett's esophagus cohort. *Cancer Epidemiology and Prevention Biomarkers*.

Hepp, D., Gonçalves, G.L. and de Freitas, T.R.O. (2015). Prediction of the damage-associated non-synonymous single nucleotide polymorphisms in the human MC1R gene. *PloS one* **10**(3):e0121812.

Herskovic, A., Martz, K., Al-Sarraf, M., Leichman, L., Brindle, J., Vaitkevicius, V., … Emami, B. (1992). Combined chemotherapy and radiotherapy compared with radiotherapy alone in patients with cancer of the esophagus. *New England Journal of Medicine* **326**(24):1593–1598.

Hollstein, M., Sidransky, D., Vogelstein, B. and Harris, C.C. (1991). p53 mutations in human cancers. *Science (New York, N.Y.)* **253**(5015):49–53. doi: https://doi.org/10.1126/science.1905840.

Hollstein, M.C., Metcalf, R.A., Welsh, J.A., Montesano, R. and Harris, C.C. (1990). Frequent mutation of the p53 gene in human esophageal cancer. *Proceedings of the National Academy of Sciences of the United States of America* **87**(24):9958–61. doi: https://doi.org/10.1073/pnas.87.24.9958.

IARC, I.A. for R. on C. *TP53 Mutation*. Available at: http://www.iarc.fr/p53. Accessed August 2001.

Ilson, D.H. (2008). Esophageal cancer chemotherapy: recent advances. *Gastrointestinal cancer research: GCR* **2**(2):85.

İmazeki, F., Omata, M., Nose, H., Ohto, M. and Isono, K. (1992). p53 gene mutations in gastric and esophageal cancers. *Gastroenterology* **103**(3):892–

896.

Iyer, R. and Dubrow, R. (2004). Imaging of esophageal cancer. *Cancer Imaging* **4**(2):125.

Jemal, A., Bray, F. and Ferlay, J. (2011). Global Cancer Statistics: 2011. *CA Cancer J Clin* **49**(2):1,33-64. doi: https://doi.org/10.3322/caac.20107.Available.

Jia, M., Yang, B., Li, Z., Shen, H., Song, X. and Gu, W. (2014). Computational analysis of functional single nucleotide polymorphisms associated with the CYP11B2 gene. *PLoS ONE* **9**(8). doi: https://doi.org/10.1371/journal.pone.0104311.

Jomrich, G., Paireder, M., Gleiss, A., Kristo, I., Harpain, L. and Schoppmann, S.F. (2017). Comparison of Inflammation-Based Prognostic Scores in a Cohort of Patients with Resectable Esophageal Cancer. *Gastroenterology research and practice* **2017**.

Kamangar, F., Chow, W.-H., Abnet, C.C. and Dawsey, S.M. (2009). Environmental causes of esophageal cancer. *Gastroenterology Clinics* **38**(1):27–57.

Kim, J.A. and Shah, P.M. (2017). Screening and prevention strategies and endoscopic management of early esophageal cancer. *Chinese clinical oncology* **6**(5).

Kimura, M., Ishiguro, H., Tanaka, T. and Takeyama, H. (2015). Advanced esophageal cancer with tracheobronchial fistula successfully treated by esophageal bypass surgery. *International journal of surgery case reports* **9**:115–118.

Kojima, T. and Doi, T. (2017). Immunotherapy for esophageal squamous cell carcinoma. *Current oncology reports* **19**(5):33.

Kolaric, K., Zupanc, D. and Zivkovic, M. (1992). Radiation alone vs radiochemotherapy (ddp+ 5fu) in locoregionally advanced esophageal cancer--interim report of a prospective randomized study. *Proc Am Soc Clin Oncol*. p. A516.

Krause, W.J. (2005). *Krause's Essential Human Histology for Medical Students*. Universal-Publishers.

Kumar, V., Abbas, A.K. and Aster, J.C. (2013). *Robbins Patologia Básica*. Elsevier Brasil.

Lehrbach, D., Nita, M. and Cecconello, I. (2003). Molecular aspects of esophageal squamous cell carcinoma carcinogenesis. *Arq Gastroenterol* **40**(4):256–261.

Leicester, U. of (2012). *FinchTV*. Available at: https://www2.le.ac.uk/offices/itservices/ithelp/my-computer/programs/finchtv.

Levine, A.J., Momand, J. and Finlay, C.A. (1991). The p53 tumour suppressor gene. *Nature* **351**(6326):453.

Li, L.-Y., Tang, J.-T., Jia, L.-Q. and Li, P.-W. (2005). Mutations of p53 gene exons 4-8 in human esophageal cancer. *World journal of gastroenterology : WJG* **11**(19):2998–3001.

Liao, Z., Cox, J.D. and Komaki, R. (2007). Radiochemotherapy of esophageal cancer. *Journal of Thoracic Oncology* **2**(6):553–568.

Lieberman, M.D., Shriver, C.D., Bleckner, S. and Burt, M. (1995). Carcinoma of the esophagus: Prognostic significance of histologic type. *The Journal of Thoracic and Cardiovascular Surgery* **109**(1):130–139. doi: https://doi.org/10.1016/S0022-5223(95)70428-0.

Lightdale, C.J. (1999). Re: Practice guidelines for esophageal cancer. *The American journal of gastroenterology* **94**(1):20–29. doi: https://doi.org/10.1111/j.1572-0241.1999.02319.x.

Lin, J., Kennedy, S.H., Svarovsky, T., Rogers, J., Kemnitz, J.W., Xu, A. and Zondervan, K.T. (2009). High-quality genomic DNA extraction from formalin-fixed and paraffin-embedded samples deparaffinized using mineral oil. *Analytical biochemistry* **395**(2):265–267.

Ljung, R., Drefahl, S., Andersson, G. and Lagergren, J. (2013). Socio-demographic and geographical factors in esophageal and gastric cancer mortality in Sweden. *PloS one* **8**(4):e62067.

Marieb, E.N. (2015). *Essential of Human Anatomy & Physiology*.

Michael H, R. and Wojciech, P. (2011). *Histology: A Text and Atlas*. Sixth.

Mohammed, M.E., Abuidris, D.O., Elgaili, E.M. and Gasmelseed, N. (2012). Predominance of females with oesophageal cancer in Gezira, Central Sudan. *Arab Journal of Gastroenterology* **13**(4):174–177.

Mullick, T. and Falk, G.W. (2001). Esophageal strictures: etiology and diagnosis. *Techniques in Gastrointestinal Endoscopy* **3**(3):128–134.

Nadeau, J.H. (2002). Single nucleotide polymorphisms: tackling complexity. *Nature* **420**(6915):517.

NCBI (2018). No Title TP53 tumor protein p53 [ Homo sapiens (human) ].

Ojima, I., Lichtenthal, B., Lee, S., Wang, C. and Wang, X. (2016). Taxane anticancer agents: a patent perspective. *Expert opinion on therapeutic patents* **26**(1):1–20.

Okuda, E., Osugi, H., Morimura, K., Takada, N., Takemura, M., Fukushima, S., … Kinoshita, H. (2001). Detection of p53 gene mutations in human esophageal squamous cell carcinomas using a p53 yeast functional assay: possible difference in esophageal carcinogenesis between the young and the elderly group. *Clinical cancer research* **7**(3):600–606.

OncologyGroup, J.E. (1993). A comparison of chemotherapy and radiotherapy as adjuvant treatment to surgery for esophageal carcinoma. *Chest* **104**:203–207.

Oren, M. (1999). Regulation of the p53 tumor suppressor protein. *Journal of Biological Chemistry* **274**(51):36031–36034.

Pflaum, J., Schlosser, S. and Müller, M. (2014). p53 family and cellular stress responses in cancer. *Frontiers in oncology* **4**:285.

Pham, P. V (2018). Medical Biotechnology: Techniques and Applications. *Omics Technologies and Bio-Engineering*. Elsevier, pp. 449–469.

Purves, D., Augustine, G.J., Fitzpatrick, D., Hall, W.C., LaMantia, A.S., McNamara, J.O. and White, L.E. (2014). Neuroscience, 2008. *De Boeck, Sinauer, Sunderland, Mass*.

Ramensky, V., Bork, P. and Sunyaev, S. (2002). Human non-synonymous SNPs: server and survey. *Nucleic acids research* **30**(17):3894–3900.

RCPath Cancer Services Working Group (2013). Dataset for the histopathological reporting of oesophageal carcinoma (2nd edition). *The Royal College of Pathologists*(261035):1–27.

Reed, C.E. (1999). Surgical management of esophageal carcinoma. *The oncologist* **4**(2):95–105.

Renehan, A.G., Tyson, M., Egger, M., Heller, R.F. and Zwahlen, M. (2008). Body-mass index and incidence of cancer: a systematic review and meta-analysis of prospective observational studies. *The Lancet* **371**(9612):569–578.

RICK (2013). *Incidence of Esophageal Cancer in Sudan*.

Rivlin, N., Brosh, R., Oren, M. and Rotter, V. (2011). Mutations in the p53 tumor suppressor gene: important milestones at the various steps of tumorigenesis. *Genes & cancer* **2**(4):466–474.

Rubenstein, J.H. and Shaheen, N.J. (2015). Epidemiology, diagnosis, and management of esophageal adenocarcinoma. *Gastroenterology* **149**(2):302–317.

Rustgi, A. and El-Serag, H. (2014). Esophageal Carcinoma. *New England Journal of Medicine* **371**(26):2499–2509. doi: https://doi.org/10.1056/NEJMc1500692.

Saeed, I.E., Weng, H., Mohamed, K.H. and Mohammed, S.I. (2014). Cancer incidence in Khartoum, Sudan: first results from the Cancer Registry, 2009–2010. *Cancer medicine* **3**(4):1075–1084.

Saunders, H.S., Wolfman, N.T. and Ott, D.J. (1997). Esophageal cancer.

Radiologic staging. *Radiologic Clinics of North America* **35**(2):281–294.

van der Schaaf, M.K., Tilanus, H.W., van Lanschot, J.J.B., Johar, A.M., Lagergren, P., Lagergren, J. and Wijnhoven, B.P.L. (2014). The influence of preoperative weight loss on the postoperative course after esophageal cancer resection. *The Journal of thoracic and cardiovascular surgery* **147**(1):490–495.

Schwarz, J.M., Cooper, D.N., Schuelke, M. and Seelow, D. (2014). Mutationtaster2: Mutation prediction for the deep-sequencing age. *Nature Methods* **11**(4):361–362.

Schwarz, J.M. and Seelow, D. *Mutation T@ster Documentation*. Available at: http://www.mutationtaster.org/info/documentation.html#bayes [Accessed: 7 April 2018].

Shaheen, O., Ghibour, A. and Alsaid, B. (2017). Esophageal Cancer Metastases to Unexpected Sites: A Systematic Review. *Gastroenterology research and practice* **2017**.

Shi, S.T., Yang, G.Y., Wang, L.D., Xue, Z., Feng, B., Ding, W., … Yang, C.S. (1999). Role of p53 gene mutations in human esophageal carcinogenesis: results from immunohistochemical and mutation analyses of carcinomas and nearby non-cancerous lesions. *Carcinogenesis* **20**(4):591–597. doi: https://doi.org/10.1093/carcin/20.4.591.

Short, M.W., Burgers, K.G. and Fry, V.T. (2017). Esophageal Cancer. *American family physician* **95**(1).

Shridhar, R., Almhanna, K., Meredith, K.L., Biagioli, M.C., Chuong, M.D., Cruz, A. and Hoffe, S.E. (2013). Radiation therapy and esophageal cancer.

*Cancer Control* **20**(2):97–110.

Sim, N.-L., Kumar, P., Hu, J., Henikoff, S., Schneider, G. and Ng, P.C. (2012). SIFT web server: predicting effects of amino acid substitutions on proteins. *Nucleic acids research* **40**(W1):W452--W457.

Smeds, J., Berggren, P., Ma, X., Xu, Z., Hemminki, K. and Kumar, R. (2002). Genetic status of cell cycle regulators in squamous cell carcinoma of the oesophagus: the CDKN2A (p16 INK4a and p14 ARF) and p53 genes are major targets for inactivation. *Carcinogenesis* **23**(4):645–655.

Snell, R.S. (2004). *Clinical Anatomy An Illustrated Reviews with Questions & Explanations*. 4th ed. Sun, B. (ed.).

Society, A.C. (2017). *Chemotherapy for Esophageal Cancer*. Available at: https://www.cancer.org/cancer/esophagus-cancer/treating/chemotherapy.html.

Society, C.C. (2018). *Side Effects of Radiation Therapy for Esophageal Cancer*. Available at: http://www.cancer.ca/en/cancer-information/cancer type/esophageal/treatment/radiation-therapy/side-effects/?region=bc#ixzz5CR5IkYYB.

Stavridi, E.S., Huyen, Y., Sheston, E.A. and Halazonetis, T.D. (2005). The three-dimensional structure of p53. *The P53 Tumor Suppressor Pathway and Cancer*. pp. 25–52.

SurveyMonkey (1999). *Sample Size Calculator*. Available at: https://www.surveymonkey.co.uk/mp/sample-size-calculator/.

Suvarna, K.S., Layton, C. and Bancroft, J.D. (2012). *Bancroft's Theory and*

*Practice of Histological Techniques E-Book*. Elsevier Health Sciences.

Taghavi, N., Biramijamal, F., Sotoudeh, M., Moaven, O., Khademi, H., Abbaszadegan, M.R. and Malekzadeh, R. (2010). Association of p53/p21 expression with cigarette smoking and prognosis in esophageal squamous cell carcinoma patients. *World journal of gastroenterology: WJG* **16**(39):4958.

Tew, W.P., Kelsen, D.P. and Ilson, D.H. (2005). Targeted therapies for esophageal cancer. *The Oncologist* **10**(8):590–601.

Theraputics, I. (2005). *BioEdit Biological Sequence Alignment Editor for Windows 95/98/NT/2000/XP*. Available at: http://www.mbio.ncsu.edu/BioEdit/bioedit.html.

Uchino, S., Saito, T., Inomata, M., Osawa, N., Chikuba, K., Etoh, K. and Kobayashi, M. (1996). Prognostic significance of the p53 mutation in esophageal cancer. *Japanese Journal of Clinical Oncology* **26**(5). doi: https://doi.org/10.1093/oxfordjournals.jjco.a023234.

Umar, S.B. and Fleischer, D.E. (2008). Esophageal cancer: epidemiology, pathogenesis and prevention. *Nature Reviews Gastroenterology and Hepatology* **5**(9):517.

Underwood, J.C.E. and Cross, S.S. (2009). *General and Systematic Pathology E-Book*. Elsevier Health Sciences.

Venselaar, H., te Beek, T.A.H., Kuipers, R.K.P., Hekkelman, M.L. and Vriend, G. (2010). Protein structure analysis of mutations causing inheritable diseases. An e-Science approach with life scientist friendly interfaces. *BMC Bioinformatics* **11**. doi: https://doi.org/10.1186/1471-2105-

11-548.

Walker, B.R. and Colledge, N.R. (2013). *Davidson's Principles and Practice of Medicine E-Book*. Elsevier Health Sciences.

Wang, C., Wang, J., Chen, Z., Gao, Y. and He, J. (2017). Immunohistochemical prognostic markers of esophageal squamous cell carcinoma: a systematic review. *Chinese journal of cancer* **36**(1):65.

Wang, L.-D., Zhou, F.-Y., Li, X.-M., Sun, L.-D., Song, X., Jin, Y., … Zhang, X.-J. (2010). Genome-wide association study of esophageal squamous cell carcinoma in Chinese subjects identifies susceptibility loci at PLCE1 and C20orf54. *Nature Genetics* **42**(9):759–763. doi: https://doi.org/10.1038/ng.648.

Wang, L., Yang, H.-Y. and Zheng, Y.-Q. (2012). Personalized medicine of esophageal cancer. *Journal of cancer research and therapeutics* **8**(3):343.

Waraich, N., Rashid, F., Jan, A., Semararo, D., Deb, R., Leeder, P.C. and Iftikhar, S.Y. (2011). Vascular invasion is not a risk factor in oesophageal cancer recurrence. *International Journal of Surgery* **9**(3):237–240. doi: https://doi.org/10.1016/j.ijsu.2010.12.002.

WEST, A.B. and MITCHELL, K.A. (2009). Vascular Disorders of the GI Tract. *Surgical Pathology of the GI Tract, Liver, Biliary Tract, and Pancreas*:185.

Wheeler, J.B. and Reed, C.E. (2012). Epidemiology of esophageal cancer. *Surgical Clinics of North America* **92**(5):1077–1087.

Zhang, H.-Z., Jin, G.-F. and Shen, H.-B. (2012). Epidemiologic differences

in esophageal cancer between Asian and Western populations. *Chinese journal of cancer* **31**(6):281.

Zhang, Y. (2013). Epidemiology of esophageal cancer. *World Journal of Gastroenterology* **19**(34):5598–5606. doi: https://doi.org/10.3748/wjg.v19.i34.5598.

Zheng, H., Wang, Y., Tang, C., Jones, L., Ye, H., Zhang, G., … Chen, S.Y. (2016). TP53, PIK3CA, FBXW7 and KRAS mutations in esophageal cancer identified by targeted sequencing. *Cancer Genomics and Proteomics* **13**(3):231–238. doi: https://doi.org/10.4172/1747-0862.1000145.

# 7.2 APPENDICES

## APPENBIX I: (Patients data)

| No. | Age categories | sex | Histological diagnosis | Immunohistochemistry status |
|---|---|---|---|---|
| **1** | 70 | M | MDLCK. SCC | negative |
| **2** | 44 | M | PD. CA | negative |
| **3** | 54 | F | WDLCK.SCC | negative |
| **4** | 55 | M | PD. SCC | negative |
| **5** | 70 | F | ADENO. CA | positive |
| **6** | 60 | F | PD. ADENO | positive |
| **7** | 40 | F | LCK. SCC | positive |
| **8** | 42 | M | WDLCK.SCC | positive |
| **9** | 70 | F | LCN. SCC | negative |
| **10** | 78 | M | SCC | positive |
| **11** | 70 | M | ADENO. CA | negative |
| **12** | 67 | M | I. SCC | negative |
| **13** | 60 | F | MD. SCC | negative |
| **14** | 65 | F | WD. SCC | positive |
| **15** | 18 | M | WD. SCC | positive |
| **16** | 40 | F | WD. SCC | positive |
| **17** | 65 | F | MD. SCC | negative |
| **18** | 85 | F | MDI. SCC | negative |
| **19** | 38 | M | MDI. ADENO | negative |
| **20** | 50 | F | PD. SCC | positive |
| **21** | 75 | F | MD. SCC | negative |
| **22** | 43 | F | SCC | positive |
| **23** | 72 | F | WD. SCC | positive |
| **24** | 70 | F | SCC | positive |
| **25** | 45 | F | WDK. SCC | negative |

| | | | | |
|---|---|---|---|---|
| **26** | 25 | M | WD. SCC | positive |
| **27** | 43 | F | WDK. SCC | negative |
| **28** | 35 | M | ADENO. CA | negative |
| **29** | 50 | M | PD. CA | negative |
| **30** | 75 | F | MD. ADENO | negative |
| **31** | 60 | F | LCK. ADENO | negative |
| **32** | 75 | M | PD. ADENO | negative |
| **33** | 82 | M | MD. ADENO | negative |
| **34** | 20 | M | SCC | negative |
| **35** | 52 | M | SCC | negative |
| **36** | 70 | F | SCC | negative |
| **37** | 61 | M | SCC | negative |
| **38** | 70 | F | SCC | negative |
| **39** | 40 | M | SCC | negative |
| **40** | 90 | F | K. SCC | positive |
| **41** | 68 | M | MDI. SCC | positive |
| **42** | 85 | F | PD. CA | negative |
| **43** | 70 | F | LCN. SCC | negative |
| **44** | 70 | F | PD. CA | negative |
| **45** | 65 | F | WDIK. SCC | negative |
| **46** | 75 | F | SCC | positive |
| **47** | 65 | F | PD. CA | negative |
| **48** | 35 | M | MD. ADENO | negative |
| **49** | 65 | F | K. SCC | negative |
| **50** | 70 | F | WD. SCC | negative |
| **51** | 85 | M/F | MDNK. SCC | positive |
| **52** | 70 | M | MD. SCC | positive |
| **53** | 60 | F | SCC | negative |
| **54** | 75 | M | WD. SCC | negative |
| **55** | 72 | F | WD. SCC | negative |

| **56** | 38 | M | WD. SCC | negative |
|---|---|---|---|---|
| **57** | 43 | F | SCC | negative |
| **58** | 70 | F | MD. SCC | negative |
| **59** | 44 | M | Infiltrating. SCC | negative |
| **60** | 65 | F | WD. SCC | negative |
| **61** | 70 | M | WD. SCC | positive |
| **62** | 60 | M | PD. SCC | negative |
| **63** | 70 | F | MDK. SCC | negative |
| **64** | 53 | M | I. SCC | negative |
| **65** | 65 | M | SCC | positive |
| **66** | 60 | F | MD.SCC | negative |
| **67** | 70 | M | WD. SCC | positive |
| **68** | 70 | F | WD. SCC | positive |
| **69** | 47 | F | SCC | negative |
| **70** | 60 | F | SCC | negative |
| **71** | 70 | M | ADENO. CA | negative |
| **72** | 50 | M | PD.CA | negative |
| **73** | 60 | F | WD. SCC | positive |
| **74** | 70 | M | PD. CA | positive |
| **75** | 30 | M | MD. SCC | negative |
| **76** | 26 | M | ADENO. CA | negative |
| **77** | 70 | M | ADENO. CA | negative |
| **78** | 35 | F | WD. SCC | positive |
| **79** | 60 | M | PD. CA | negative |
| **80** | 37 | F | MD. SCC | positive |
| **81** | 70 | F | MD. SCC | positive |
| **82** | 57 | F | MD. SCC | positive |
| **83** | 70 | M | SCC | negative |
| **84** | 70 | M | SCC | negative |
| **85** | 85 | F | SCC | negative |

| | | | | |
|---|---|---|---|---|
| **86** | 75 | F | SCC. In Situ | negative |
| **87** | 50 | F | WD.SCC | negative |
| **88** | 60 | F | MD. SCC | negative |
| **89** | 65 | F | SCC | negative |
| **90** | 45 | F | SCC | negative |
| **91** | 60 | F | SCC | negative |
| **92** | 75 | F | SCC | negative |
| **93** | 93 | M | SCC | positive |
| **94** | 55 | F | SCC | negative |
| **95** | 37 | F | SCC | negative |
| **96** | 80 | M | MD. SCC | positive |
| **97** | 35 | M | PD. ADENO | negative |
| **98** | 82 | F | MD. SCC | negative |
| **99** | 70 | F | WDI. SCC | negative |
| **100** | 60 | M | WDI. ADENO | negative |
| **101** | 40 | F | MDI. SCC | negative |
| **102** | 70 | F | WD.SCC | negative |
| **103** | 38 | M | WD.SCC | negative |
| **104** | 75 | F | MDI. SCC | negative |
| **105** | 60 | F | I. SCC | negative |
| **106** | 70 | F | MD.SCC | positive |
| **107** | 60 | M | WD.SCC | positive |
| **108** | 89 | M | WD.SCC | negative |
| **109** | 63 | F | ADENO. CA | negative |
| **110** | 60 | M | MD. SCC | negative |
| **111** | 65 | F | WD.SCC | negative |
| **112** | 60 | F | MD.SCC | negative |
| **113** | 80 | F | SCC | Negative |
| **114** | 72 | M | UDCA | negative |
| **115** | 60 | M | MD. SCC | positive |

| **116** | 45 | F | MD.SCC | negative |
|---|---|---|---|---|
| **117** | 45 | F | MD. SCC | negative |
| **118** | 60 | M | INK.SCC | negative |
| **119** | 64 | M | INFILTRATIVE. CA | negative |
| **120** | 75 | M | PD. CA | negative |
| **121** | 40 | F | WD. SCC | negative |
| **122** | 55 | F | SCC | negative |
| **123** | 70 | M | MD. SCC | negative |
| **124** | 49 | F | WD. SCC | negative |
| **125** | 70 | M | MD. SCC | negative |
| **126** | 60 | M | UNDIFF. CA | negative |
| **127** | 40 | F | PD.SCC | negative |
| **128** | 68 | M | WD. SCC | negative |
| **129** | 40 | F | WD. SCC | negative |
| **130** | 38 | M | MD. SCC | positive |
| **131** | 45 | F | WD. SCC | negative |
| **132** | 55 | F | SCC | positive |
| **133** | 70 | F | PD. SCC | negative |
| **134** | 54 | F | WDLCK.SCC | negative |
| **135** | 50 | F | PD. SCC | negative |
| **136** | 50 | F | SCC | negative |
| **137** | 65 | F | MD. SCC | negative |
| **138** | 55 | F | SCC | negative |
| **139** | 55 | F | SCC | negative |
| **140** | 55 | F | SCC | negative |
| **141** | 75 | F | SCC | negative |
| **142** | 75 | F | SCC | negative |
| **143** | 36 | F | SCC | positive |
| **144** | 70 | F | SCC | positive |
| **145** | 60 | F | SCC | negative |

| | | | | |
|---|---|---|---|---|
| **146** | 76 | F | SCC | negative |
| **147** | 60 | F | SCC | negative |
| **148** | 80 | M | SCC | positive |
| **149** | 45 | F | SCC | negative |
| **150** | 55 | F | SCC | negative |
| **151** | 70 | F | SCC | negative |
| **152** | 60 | F | SCC | negative |
| **153** | 85 | F | SCC | Negative |
| **154** | 54 | M | SCC | negative |
| **155** | 27 | F | SCC | negative |
| **156** | 70 | F | WD.SCC | negative |
| **157** | 51 | M | SCC | negative |
| **158** | 65 | F | SCC | positive |
| **159** | 60 | M | SCC | positive |
| **160** | 60 | F | SCC | negative |
| **161** | 55 | F | SCC | negative |
| **162** | 27 | F | SCC | negative |
| **163** | 50 | F | SCC | negative |
| **164** | 65 | F | SCC | positive |
| **165** | 70 | F | SCC | positive |
| **166** | 70 | F | SCC | negative |
| **167** | 75 | M | SCC | positive |
| **168** | 50 | F | SCC | negative |
| **169** | 70 | F | SCC | negative |
| **170** | 65 | F | SCC | negative |
| **171** | 60 | F | PD.SCC | positive |
| **172** | 50 | M | SCC | negative |
| **173** | 67 | M | PD.C | Negatiive |
| **174** | 60 | M | MD.SCC | Negatiive |
| **175** | 60 | F | MD.SCC | positive |

| **176** | 66 | M | MD.SCC | Negatiive |
|---|---|---|---|---|
| **177** | 50 | F | WD.SCC | positive |
| **178** | 60 | F | MD.SCC | Negative |
| **179** | 86 | F | MD.SCC | positive |
| **180** | 80 | F | MD.SCC | positive |
| **181** | 60 | F | MD.SCC | positive |
| **182** | 60 | M | SCC | Negative |
| **183** | 60 | M | SCC | positive |
| **184** | 65 | F | PD.SCC | Negative |
| **185** | 55 | F | PD.SCC | positive |
| **186** | 70 | M | ADENO | Negative |
| **187** | 60 | M | MD.SCC | negative |
| **188** | 60 | F | MD.SCC | positive |
| **189** | 60 | F | SCC | negative |
| **190** | 90 | M | SCC | positive |
| **191** | 40 | F | MD.ADENO | negative |
| **192** | 60 | M | ADENO.CA | negative |
| **193** | 29 | F | SCC | positive |
| **194** | 68 | M | NK.SCC | negative |
| **195** | 40 | F | SCC | negative |
| **196** | 46 | M | MDK.SCC | negative |
| **197** | 56 | F | ADENO.CA | negative |
| **198** | 52 | F | SCC | positive |
| **199** | 75 | F | MDK.SCC | negative |
| **200** | 70 | M | ADENO.CA | Negative |
| **201** | 60 | F | MDNK.SCC | Negative |
| **202** | 60 | F | SCC | negative |
| **203** | 72 | F | WDSCC | positive |
| **204** | 60 | M | SCC | negative |

# Appendix II: (Solutions)

**1% eosin**

| | |
|---|---|
| Eosin | 1 g |
| DW | 100 ml. |

**Mayer's hematoxylin**

| | |
|---|---|
| Hematoxylin | 1 g |
| Distilled water | 1000 ml |
| Potassium or ammonium alum | 50 g |
| Sodium iodate | 0.2 g |
| Citric acid | 1 g |
| Chloral hydrate SLR | 50 g or |
| Chloral hydrate AR | 30 g. |

**Intron Mastermix: Catalog no: 25178**

**Double distilled water**

**Primers (Macrogen)**

**Absolute alcohol:** (99.89%)

**Ethidium bromide:** stock= (10 mg/ml), working= (5µl stock/100 ml gel).

### 10X running buffer TBE

| Tris base | 108.0g |
|---|---|
| Boric acid | 55.0g |
| Na4EDTA | 9.34g |

Add H2O to give a final volume of 1 litre

### Materials and instrument used for immunohistochemistry processing and staining:

Disposable gloves

Microtome blades

Positively charged slide

Cover glass

Dry oven

Water bath

Embedding center

Coplin jar

Humidity chamber

Ethanol (100%, 90%, 70%, 50%)

Mayer s haematoxylin (DW, K or ammonium alum, sodium iodated, citric acid, chororal hydrate)

Reaction buffer

Primary antibody (EBV)

Tris EDTA buffer (PH9)

Phosphate buffer saline (PH7.4)

Peroxides blocker (3% hydrogen peroxide in methanol)

Secondary antibody

DAB (3, 3 di amino benzidin tetra hydrochloride) substrate solution

Bluing Reagent (0.1MLi2 CO3, 0.5 M Na2CO3)

Xylene

DPX mounting media

# Appendix III: (Histopathology Slides).

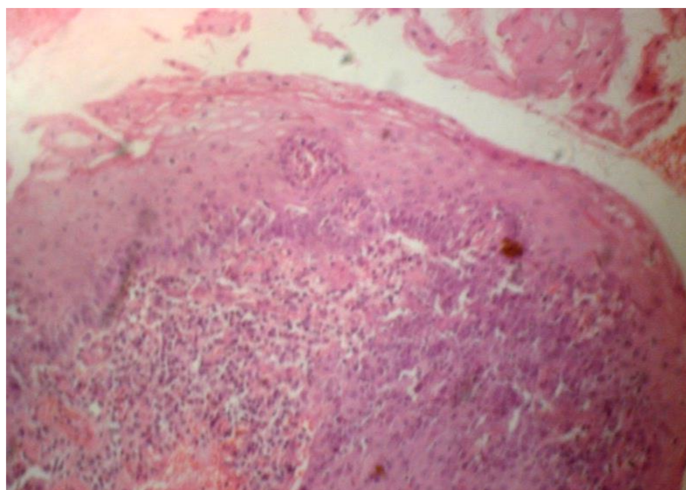


**Fig. 16**. Invasive SCC. H&E x 100.

The histopathological features of SCC showed some variations. Squamous epithelium commonly exhibited slight to moderate dysplasia of basal cells with occasional high grade (intraepithelial) dysplasia (Fig. 2 & 3).

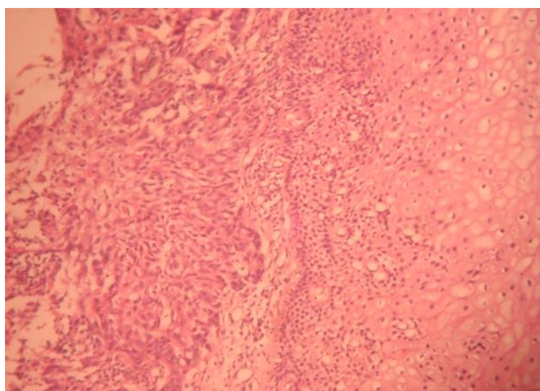


**Fig. 17** Dysplasia of basal cells with Subepithelial neoplastic cells. HE x 40.

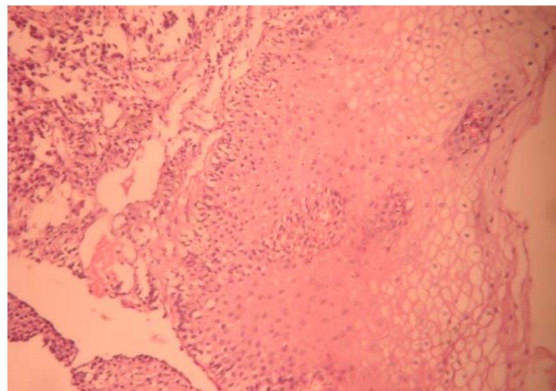


**Fig. 18** Dysplasia of basal cells with Subepithelial neoplastic cells. HE x 40.

Dysplastic cells appeared hyperchromatic, pleomorphic with nuclear enlargement with or without vesiculation. Dysplasia was associated with irregular border with downward projections into adjacent tissue (Fig. 4&5).

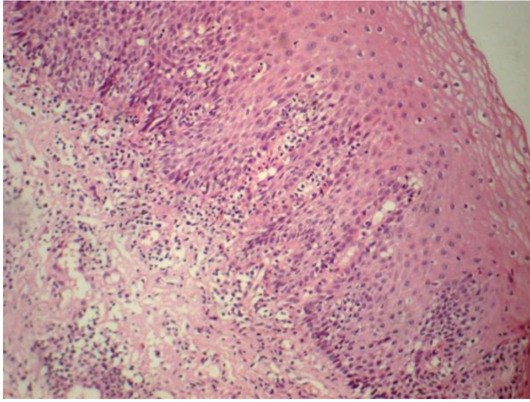


**Fig. 19** Downward projections of basal cells. HE x 100.

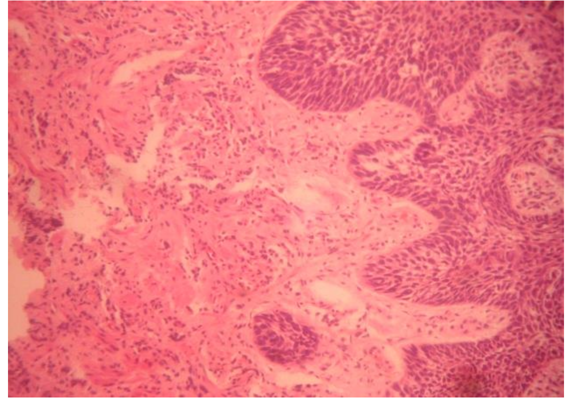


**Fig. 20** Downward projections of basal cells. HE x 100

Neoplastic cells exhibited different patterns. Some were arranged as sheets of cells, proliferating cells with trabecular, papillary, glandular or cord like pattern or as cell nests separated by scanty or dense stroma. The infiltrating neoplastic cells were dense in some sections and scattered in others (Fig. 6-.10).

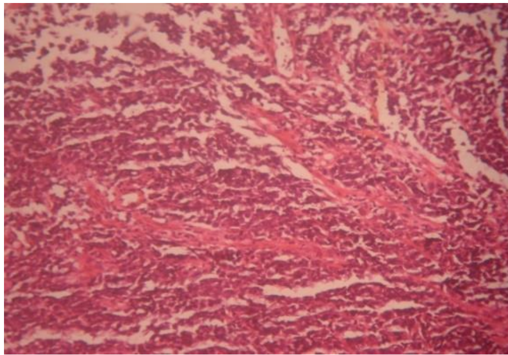


**Fig. 21** Trabecular and cord-like Pattern of neoplastic cells. HE x40

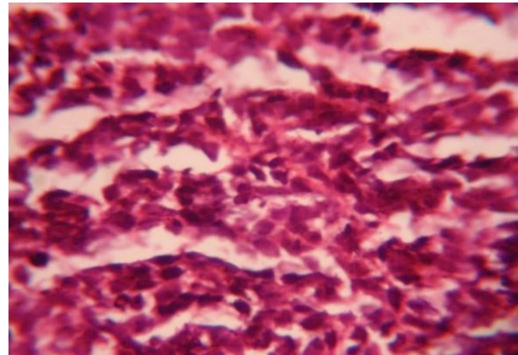


**Fig.22** Higher magnification of Fig.6. HE x 400

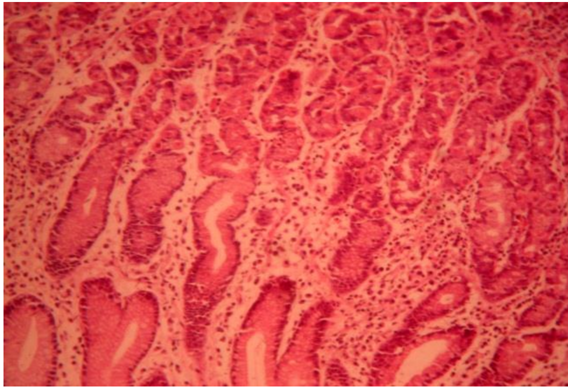


**Fig. 23.** Papillary-like Pattern of neoplastic cells. HE x40

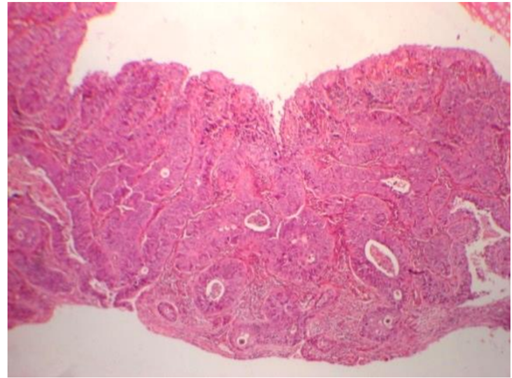


**Fig.24.** cord-like pattern of neoplastic cells. HE x 40

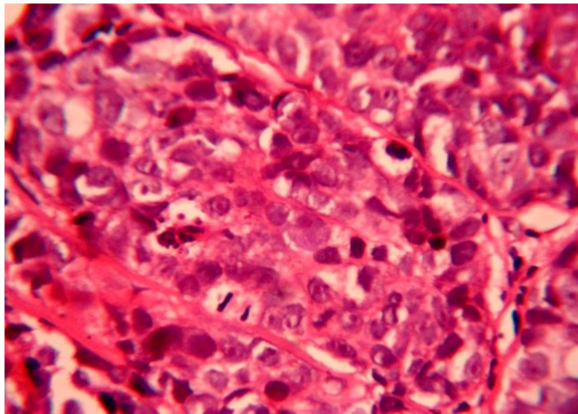


**Fig. 25.** Packed neoplastic cells separated by thin strands. He x 400.

In some sections intact squamous epithelium was seen with low basal dysplasia; tumor cells seen infiltrating underlying tissue (Fig. 11 & 12)

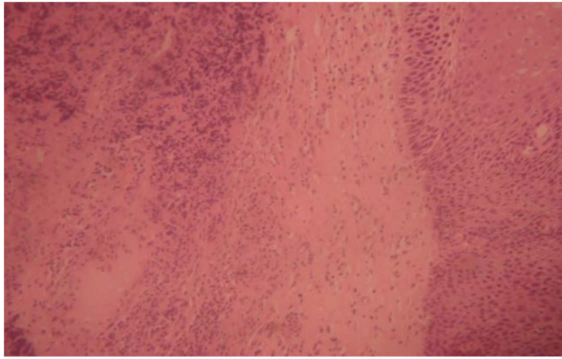


**Fig. 26.** Intact squamous epithelium with basal dysplasia. He x 40.

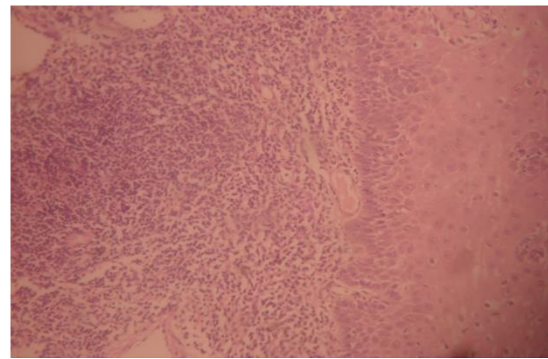


**Fig. 27.** Intact squamous epithelium with basal dysplasia. He x 40.

Differentiated neoplastic cells could be seen showing epithelial keratinization of forming large squamous pearls. In some profiles squamous cells were vacuolated with pyknotic nuclei or no nuclei, forming network of empty cells. In some sites the proliferating keratinocytes appear large with intercellular bridges (Fig.13-17)

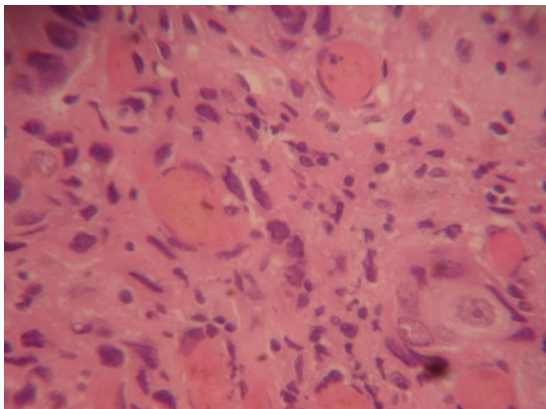
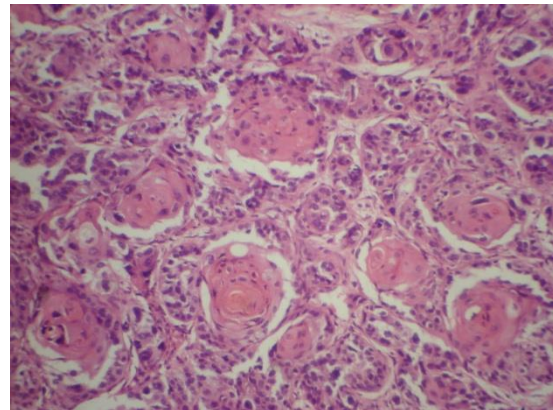


**Fig. 28.** SCC,Epithelial keratinization HE x100.



**Fig, 29.** SCC, Multiple epithelial pearls. HE x100.

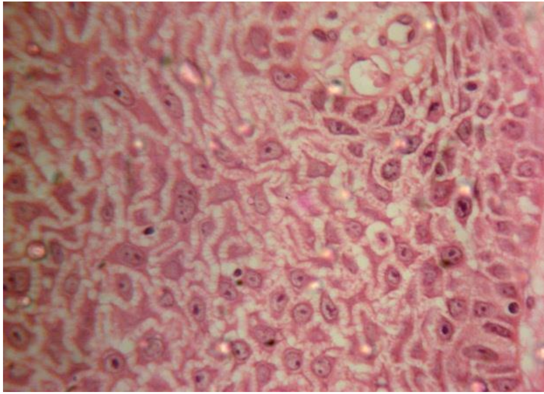


**Fig. 30.** SCC, keratinocytes with intercellular bridges. HE x 100.

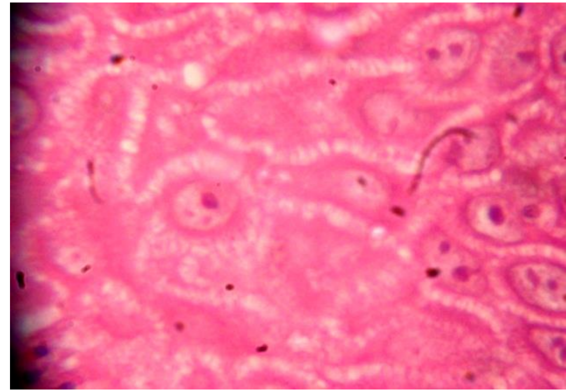


**Fig. 31.** SCC. Intercellular bridges HE x 1000.

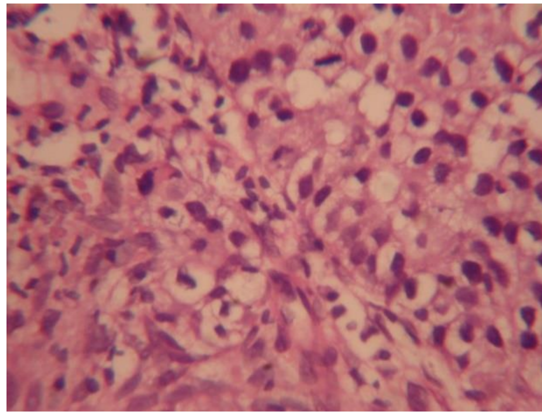


**Fig.32.** SCC vacuolated cells with pyknotic nuclei. HE x 100.

Squamous cells were seen forming groups of enlarged vacuolated cells with pagetoid appearance. In one case the tumor cells appear to expand along the free front opposite to the basal epithelium exhibiting what looks like an expansile growth pattern (18-20).

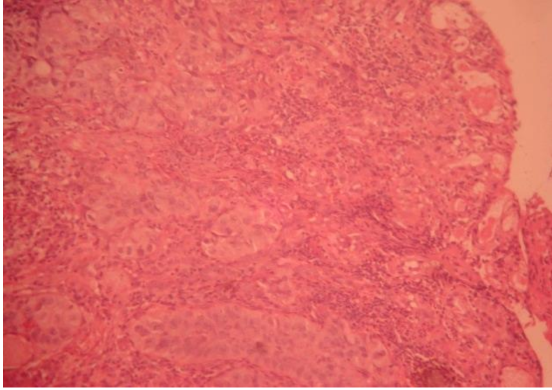


**Fig. 33.** Enlaged vacuolated cells, Pagetoid appearance. HE x 40.

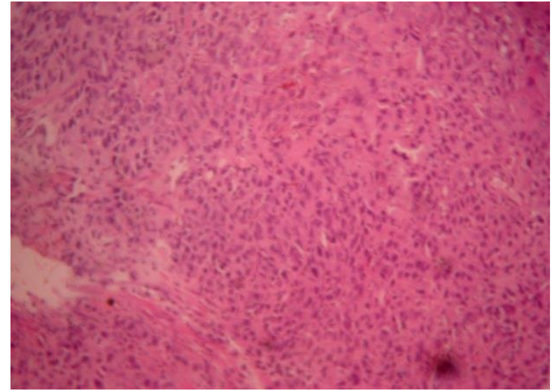


**Fig. 34.** Enlarged cells, pagetoid appearance. HE x 40.

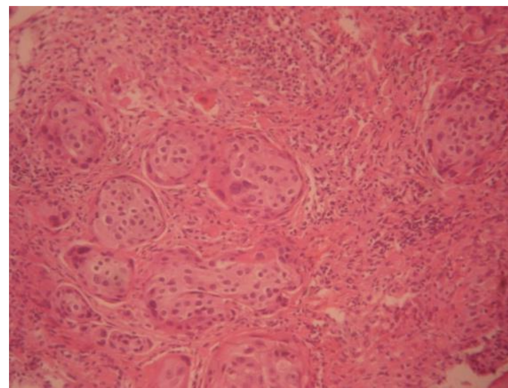


**Fig.35.** SCC Groups of vacuolated cells. HEx100.

Many sections showed fragments of tissue featuring pleomorphic hyperchromatic neolastic cells with evidence of keratinzation. Some cells appear to fuse together forming giant-like cells. In some sections necrotic changes were noticed and in others regular globular structures were seen. Presence of bacterial colonies was suspected (Fig. 21-24).

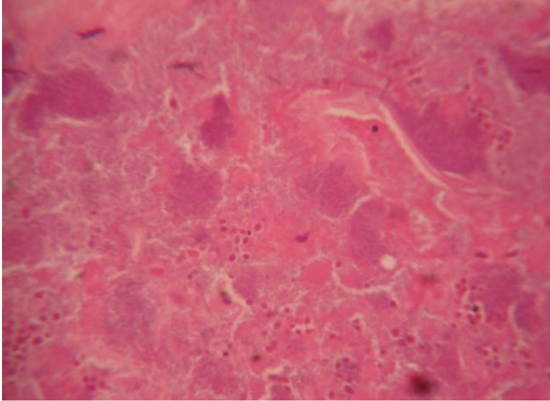


**Fig. 36.** SCC. Necrotic tumor tissue HE x 100.

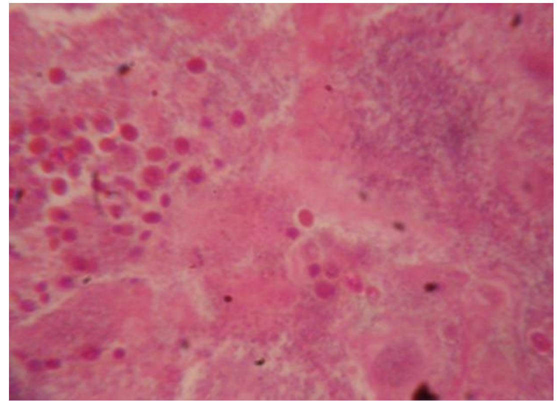


**Fig. 37.** SCC. Necrotic tumor tissue with globules. HE x 100.

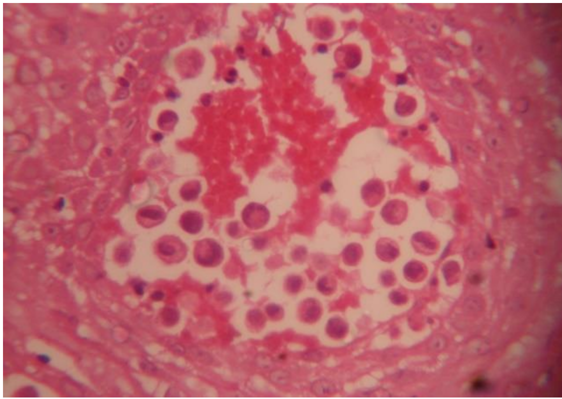


**Fig. 38.** Higher magnification of H&E x 400.
H&E x 400.

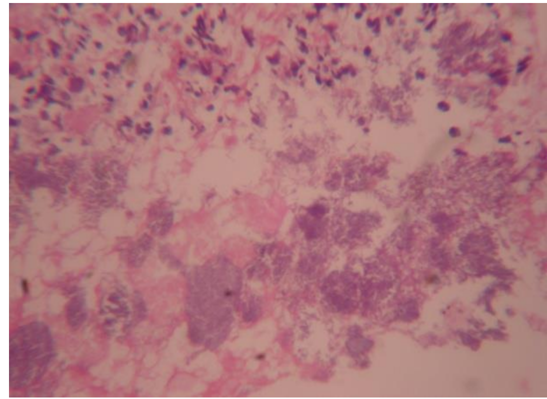


**Fig. 39.**SCC. Suspected bacterial Colonies.

In poorly differentiated (PD) tumors, the cells may appear small dense or loosely coherent (small cell carcinoma). The cells may be small and mistaken for lymphocytes or may be small pleomorphic and spindle shaped or may form clear cells (**Fig, 25- 28**).

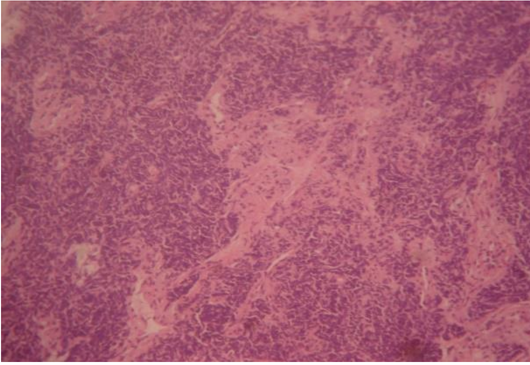


**Fig.40.** Small cell carcinoma H&E x 40.

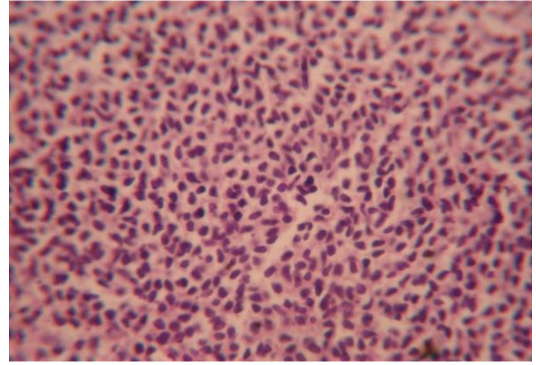


**Fig.41.**Small cell carcinoma, cells resemble lymphocytes. H&E x 100

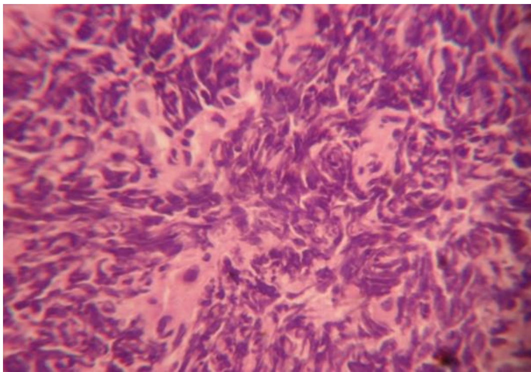


**Fig. 42.** PDSCC. Spindle shaped cells HE x 100**.**

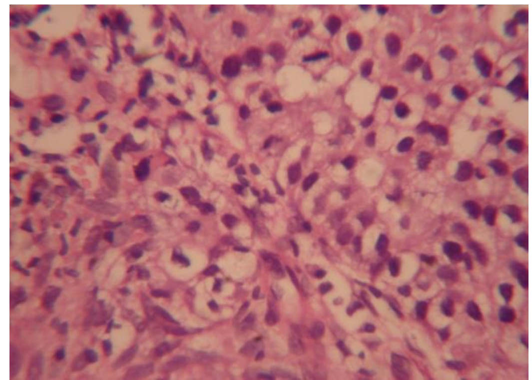


**Fig. 43.** PDSCC with clear cells HE x 400.

Anaplasia with bizarre cells and mitotic figures were seen. Many sections exhibited poorly differentiated cells with tendency to form glandular structures or tubules. Cells appeared vacuolated with more than one nucleoli (Fig. 29, 30).

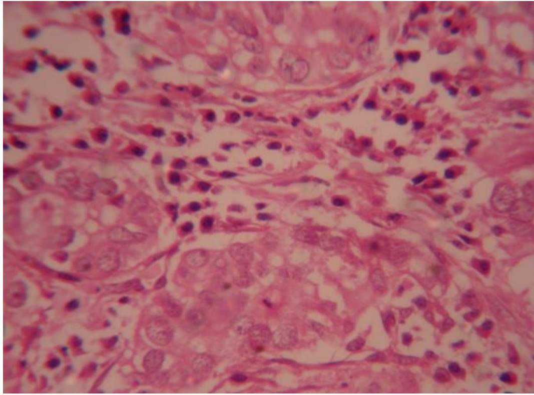


**Fig. 44.** SCC Tendancy to form Glandular structures. H&E x 100.

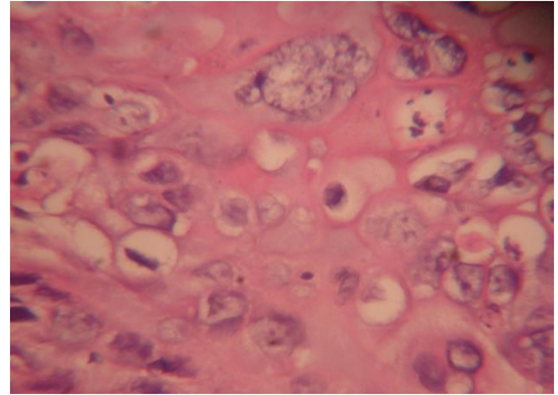


**Fig. 45.** SCC Cellular pleomorphism H&E x 400.

Round or spindle shaped hyperchromatic neoplastic cells were observed surrounding rather differentiated squamous cells (PDSCC). Some cells formed islands surrounded by scanty or rich stroma. In other sections stromal tissue predominated with few scattered squamous cells (pseudosarcomatous SCC) (**Fig. 31-34**).

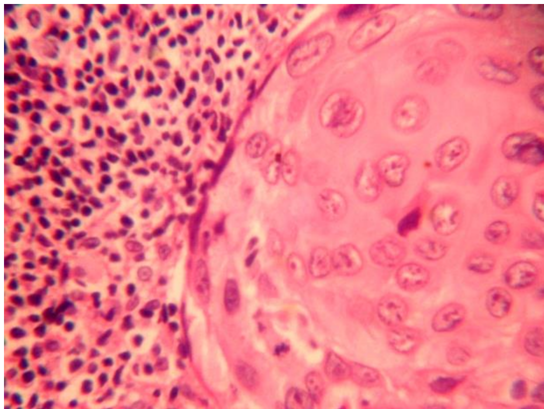


**Fig. 46.** PDSCC. H&E x 400.

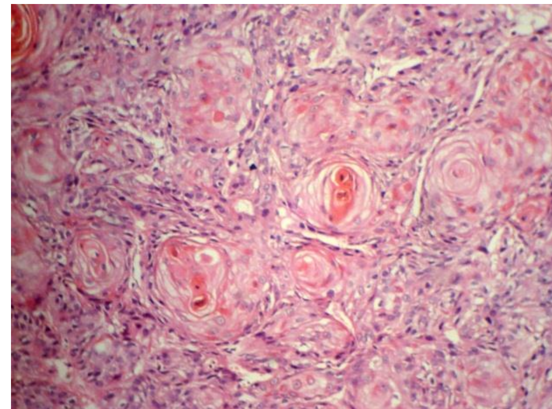


**Fig. 47.** SCC Cells nests Surrounded by scnty stroma. H&E x 100.

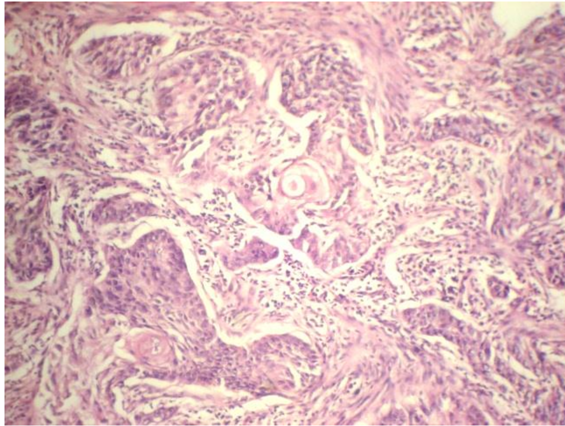


**Fig. 48.** SCC Cells nests Surrounded by wide stroma. H&E x 100.

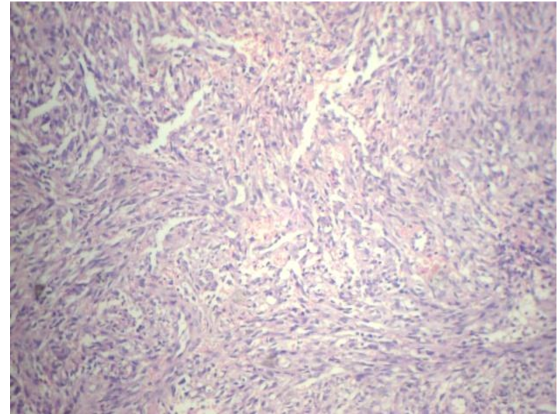


**Fig. 49.** Pseudosarcomatous SCC H&E x 40.

Sections of adenocarcinoma (AC) showed regular or irregular glandular (gastric or intestinal type) or cystic structures. The gland cells mostly had basal nuclei and appeared as mucous glands. The stroma may show round inflammatory cells (Fig. 35 – 40).

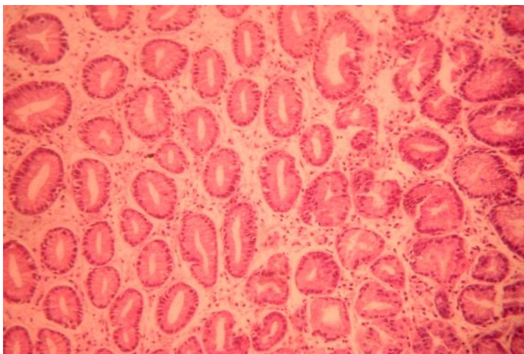


**Fig. 50.** Adenocarcinoma H&E x40.

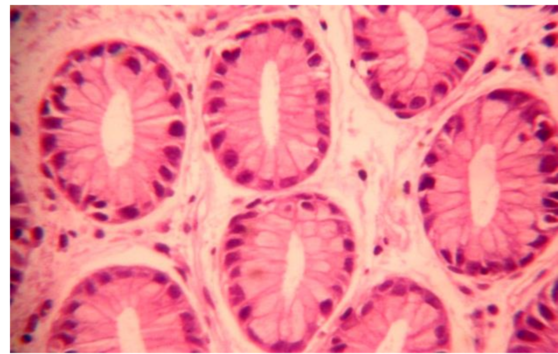


**Fig. 51.** Adenocarcinoma H&E x100.

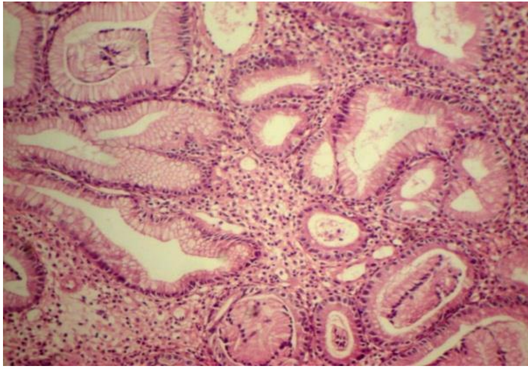


**Fig. 52.** Cystic AC H&E x 40.

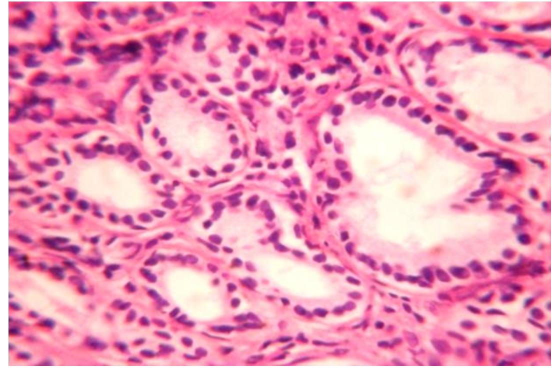


**Fig. 53.** Cystic AC H&E x 100

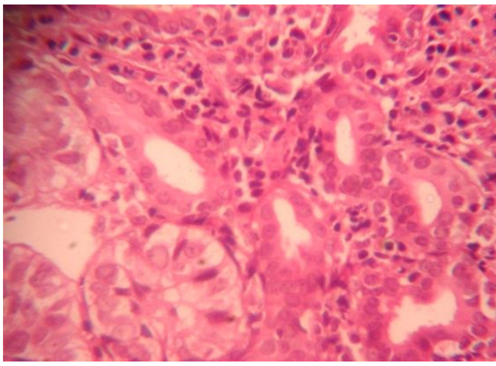


**Fig. 54.** AC Resembling gastric Mucoa. H&E x 40.

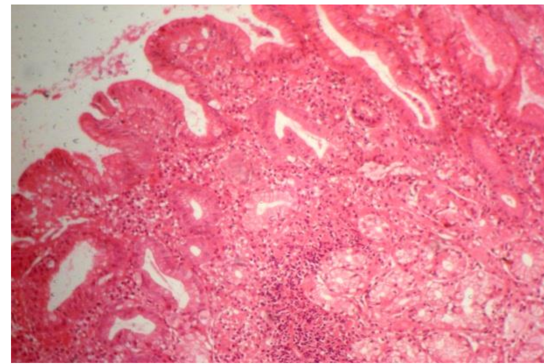
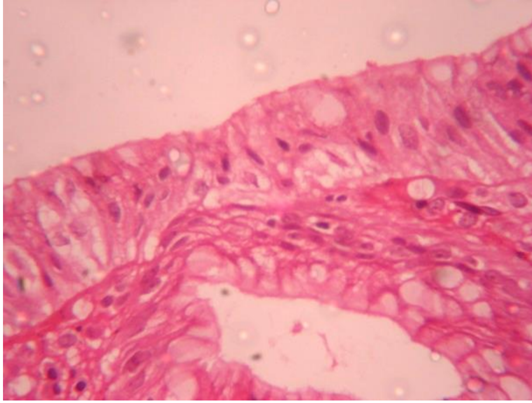


**Fig.55.** Higher magnification of Fig. 39. H&E x 100.Mucoa. H&E x 40.

In one section of moderately a differentiated SCC a tissue fraction appeared to be lined with pseudostratified columnar cells resembling Respiratory epithelium (Fig 41, 42) and numerous underlying glands with basal nuclei and foamy cytoplasm (Fig. 43, 44)



**Fig, 56.**

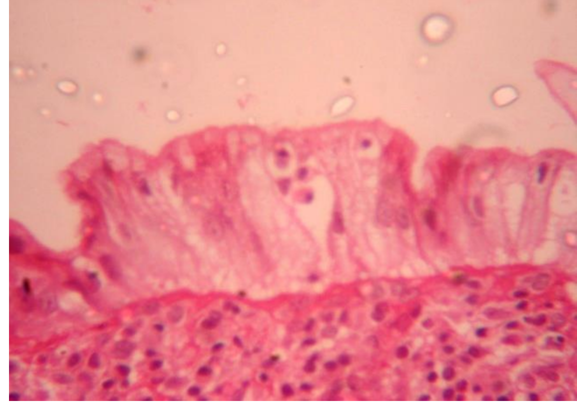


**Fig. 57.**

Both figures from SCC section. Tissue fraction lined by respiratory-like pseudostratified columnar epithelium. HE x 100

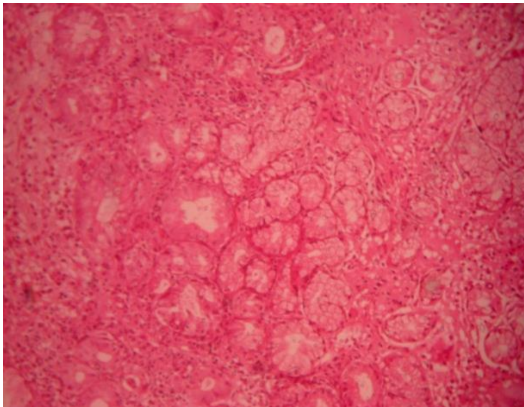


**FIG. 58.** Sub-epithelial glands HE x 40.

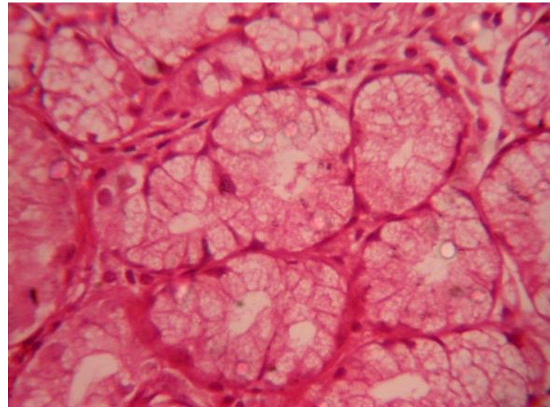


**Fig. 59.** Sub-epithelial glands.

Twenty two percent of the tumors showed desmoplasia. Epithelial pearls were frequently seen mostly in well differentiated tumors. Inflammatory cells were observed in many sections either as chronic inflammatory infiltrate or mixed with acute inflammatory cells. In few sections PMN neutrophils were predominant and were seen stuffing blood vessels (Fig. 45). The cellular infiltration was noticed in the stroma and between neoplastic cells. Occasionally neutrophils were found scattered in the surface squamous epithelium. Haemorrhage was not uncommon and in some sections was extensive (Fig. 46, 47).

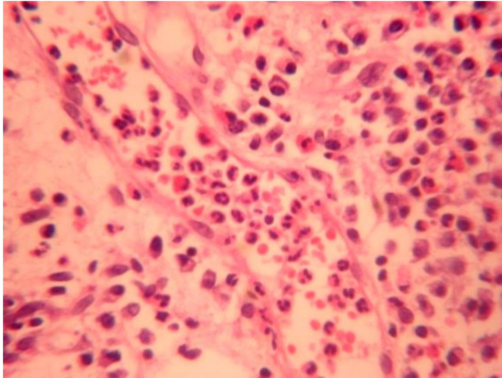


**Fig. 60.** Neutrophils in tumor tissue and blood vessel. H&E x 100.

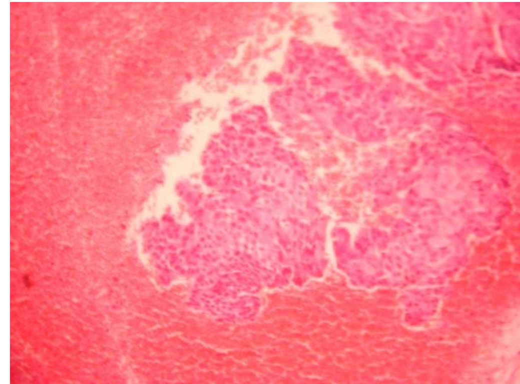


**Fig. 61.** Tumor cells in a pool of blood. H&E x 40.

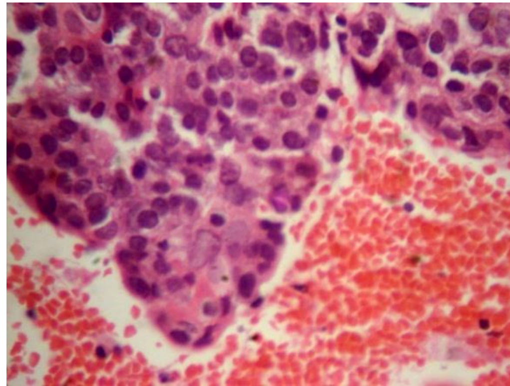


**Fig. 62.** Higher magnification of Fig, 49.HE x 400.