"ESTIMATING PREVALENCE OF SOX-2 EXPRESSION IN CERVICAL SQUAMOUS CARCINOMAS – A HOSPITAL BASED CROSS SECTIONAL STUDY"

By

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Sub: Institutional Ethical Clearance for the study.

With reference to the above, we wish to inform you that your proposed research project titled **"ESTIMATING PREVALENCE OF SOX-2 EXPRESSION IN CERVICAL SQUAMOUS CARCINOMAS – A HOSPITAL BASED CROSS SECTIONAL STUDY"**, is ethical and justifiable. The proposed research project has been cleared by the JNMC Institutional Ethics Committee on Human Subjects Research.

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ABSTRACT

"ESTIMATING PREVALENCE OF SOX-2 EXPRESSION IN CERVICAL SQUAMOUS CARCINOMAS – A HOSPITAL BASED CROSS SECTIONAL STUDY"

Background and objectives: Cervical cancer is the most common cancer (6 -29 % of all cancers) in females of developing countries like India. In the year 2018, there were 569,847 new cases of cervical cancer and 311,365 deaths worldwide making it the most common gynaecological malignancy worldwide. In the same year in India, there were 96,922 new cases of cervical cancer and 60,078 deaths. According to the WHO criteria the three categories of invasive carcinomas are squamous cell carcinomas, adenocarcinomas and others. There are various types of squamous cell carcinoma like keratinizing, non-keratinizing, papillary, etc. all of which are included in the study. Squamous cell carcinoma is the cancer of the exo-cervix presenting as an ulcerated or exophytic lesion caused by HPV, smoking, low socioeconomic environment. HPV is considered to be responsible for as much as 90% of cervical carcinomas. There is a general paucity of studies describing correlation of SOX-2 and histological grading in cervix. Thus, the aim of this study is to solve this discrepancy and evaluate the role of Sox-2 in grading the tumour and as an adjuvant prognostic marker.

Methods: In the present study, 50 histopathologically diagnosed cases of cervical squamous cell carcinomas were studied from the time period January 2021 to December 2021 at Dept of Pathology, JNMC, Belagavi. Most of the cases (90%) in this study were punch biopsies, 3 hysterectomy and 1 endocervical curettage specimens were also included. Paraffin embedded blocks of all 50 cases were subjected to immunohistochemical staining for SOX2 and its result was correlated with clinicopathological parameters. The peak incidence was between 55-64 years

with the mean and median age of 53 years and 55 years respectively. Most of the cases were moderately differentiated carcinomas according to Broder's grading system.

Results: 98% or 48 out of 50 cases of SCC cervix in this study have shown positivity for SOX2 immunostaining. However, cases varied in intensity of staining and percentage positive tumor cells. Overall IHC expression score was predominantly weak seen in 42% of the cases. There was no significant correlation found between IHC score and age or between Broder's grade and percentage of positive cells or intensity. No correlation was found between Broder's grade and IHC even when each grade group was evaluated individually.

Conclusion: This study evaluated SOX2 expression in 50 cervical squamous cell carcinomas and the expression was correlated with histological parameters like Broder's grading. The percentage of tumor cells were determined along with intensity of staining and a final IHC score was given. 48 (96%) of the 50 cases showed positivity for SOX2 expression with 4% negative, 42% weak, 28% medium and 26% with strong expression. No correlation was found between Broder's grade for differentiation and SOX2 expression.

Key words: SOX2 marker, immunohistochemistry, cervical carcinomas, squamous cell carcinoma.

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INTRODUCTION

In 2020, globally cervical carcinoma accounted for 3.4% of the deaths from cancer in females[1]. This made it the 4th leading cause of death globally with high morbidity and mortality in developing countries due to lack of HPV vaccination[1]. In India, cervical carcinoma accounts for 29% of all the cancer incidence in females between the age of 30 and 69 years making it a significant contributor to morbidity and mortality[2].

Lack of HPV vaccination program coupled with poor socioeconomic status, high number of child birth, sexually transmitted infections result in high HPV infections[2]. E6 and E7 oncoproteins from HPV bind P53 and RB respectively resulting in unregulated cell cycle and propagation of DNA errors. Cervarix and Gardasil are the vaccines approved by the FDA against HPV[3].

SOX2, a transcription factor containing the HMG domain from the SRY gene which plays an important role in sex determination and neural embryogenesis[4]. SOX2 plays an important role in maintaining pluripotency and self-renewal of cancer stem cells making it a potential marker for cancer stem cells[5]. These stem cells play an important role in tumour tumour initiation, aggressiveness by mechanisms like lineage plasticity, anti-apoptotic signalling, immune surveillance, epithelial to mesenchymal transition making them a potential target for anti-cancer therapies[5].

SOX2 is also expressed in other carcinomas like pancreas, breast, gastric, lung[6]. It was associated with aggressive behaviour in stage 1 lung adenocarcinomas and breast carcinomas[7]. In the cervix SOX2 is expressed in 20% of the normal cervix and 75% of squamous cell carcinoma cases[8]. It is found that at the transformation

zone expression changes from SOX17 in columnar epithelium to SOX2 in squamous epithelium[9]. This change of expression is also seen when the epithelial cell is infected with HPV resulting in squamous epithelium and increased SOX2 expression[8].

This research study was undertaken to study the expression of SOX2 by immunohistochemistry (IHC) in histopathologically diagnosed cases of squamous cell carcinoma (SCC) cervix. Correlation of SOX2 expression with Broder's histopathological grading in cervical cancer was also studied.

AIMS AND OBJECTIVES

Primary objective: To estimate prevalence of SOX-2 expression in cervical squamous cell carcinomas.

Secondary objective: To correlate expression of SOX-2 in cervical squamous cell carcinomas with histological grading.

REVIEW OF LITERATURE

Embryology

At the fifth week of development, the embryo develops a thickened area of mesothelium on the mesonephros resulting in the formation of a bulge called the gonadal ridge[10]. This results in the formation of indifferent gonads which based on the presence of XX or XY chromosome differentiates into the testis or ovary[10].

In a female embryo, the mesonephric ducts regress and paramesonephric ducts develop because of absence of testosterone and Mullerian inhibiting substance[11]. The paramesonephric ducts fuse to form the uterovaginal primordium[11]. This structure contains mesoderm which eventually forms the endometrium and myometrium[12]. The unfused ends of the paramesonephric ducts give rise to fallopian tubes and the caudal ends of these tubes give rise to the cervix and vagina[12].

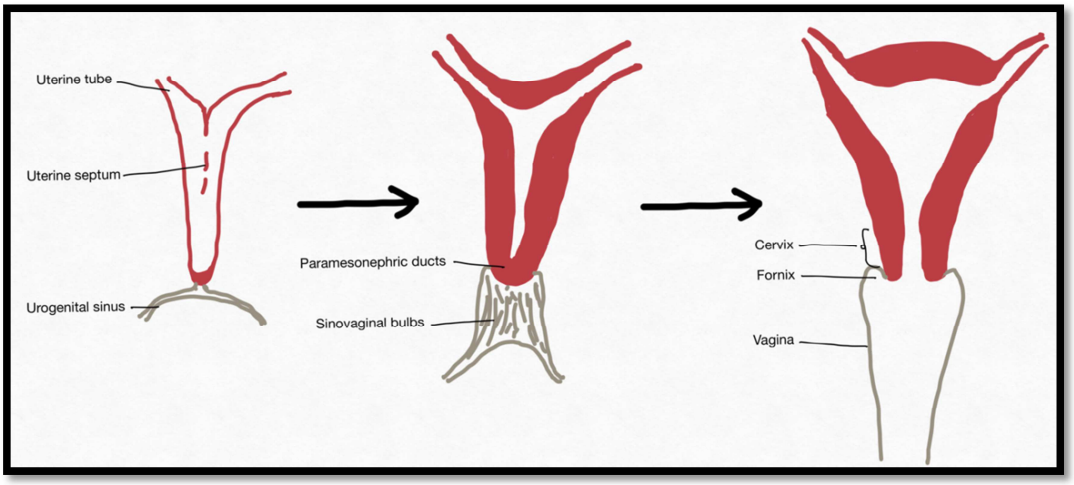


Figure 1 – Uterus, embryology[12].

Anatomy

In the neonatal period, the cervix occupies a large proportion of the length of the uterus with cervico-fundal ratio being upto 5:1[13]. This ratio is 1:1 at 13 years of age and reverses with puberty[13].

Cervix is fibromuscular organ located at the terminal end of the uterus measuring 2.5cm to 3cm and functions to link the endometrial cavity to vagina[14]. The superior part of the cervix attached to the uterus is the supra-vaginal portion located above the vaginal vault[15] . The remainder of cervix lies below the vault in the vagina called vaginal portion or portio vaginalis[12]. The part of the cervix related to the endocervical canal is the endocervix and the part seen in the vagina via colposcopy is ectocervix[15]. The opening of endometrium into the endocervical canal is internal os and opening of endocervical canal into exocervix is external os[15]. The exocervix, seen via colposcope is circular in nulliparous women and divided into anterior and posterior lips in parous women[14]. The endocervical canal has palmate folds called arbor vitae uteri[14].

The supra-vaginal part of the cervix is separated from the bladder anteriorly by parametrium and is related superiorly to the uterus, posteriorly to the rectouterine pouch containing rectum and inferiorly to the vaginal part of the cervix[16].

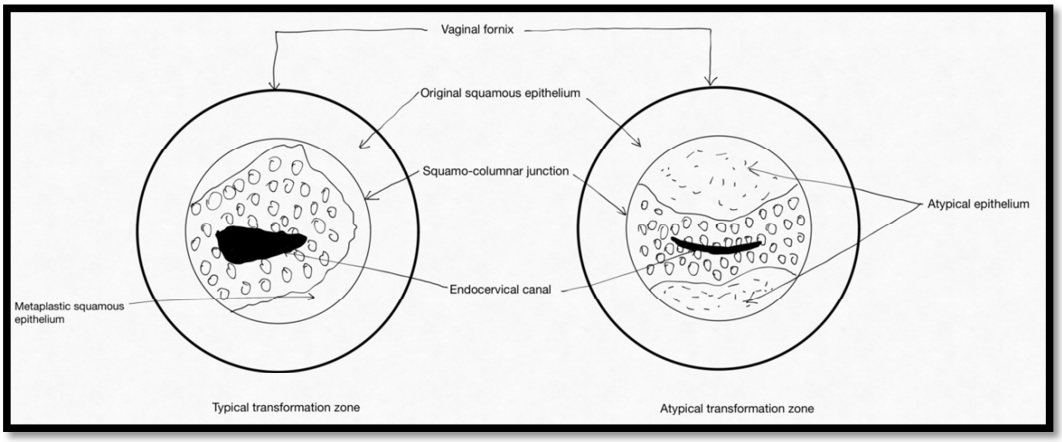


Figure 2 – Colposcopy of cervix demonstrating the transformation zone[15].

Blood supply

The internal iliac artery gives rise to the uterine artery which divides and supplies the supra-vaginal part of the cervix. The lower cervix is supplied by the vaginal artery[17]. The veinous drainage is in ureteric veins via a plexus in the broad ligament[16].

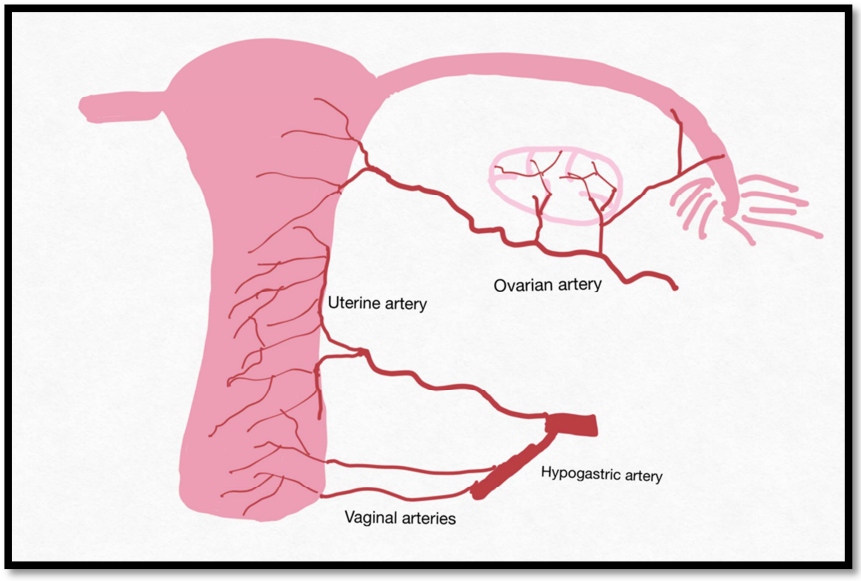


Figure 3 – Blood supply of cervix[15].

Lymphatic drainage

The internal iliac artery gives rise to the uterine artery which divides and supplies the supra-vaginal part of the cervix. The lower cervix is supplied by the vaginal artery[17]. The veinous drainage is in ureteric veins via a plexus in the broad ligament[16].

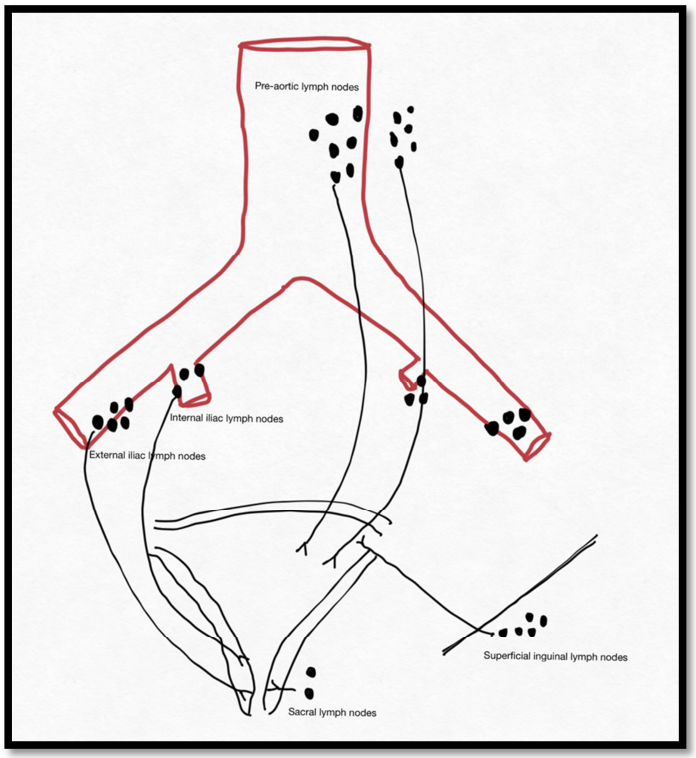


Figure 4 - Lymphatic drainage of cervix[16].

Histology

The ectocervix which is the outer surface of the vaginal portion is covered by stratified squamous epithelium while the endocervix is lined by mucin secreting columnar epithelium[3]. The junction between the squamous epithelium of the exocervix and the glandular endocervical mucosa is called the squamocolumnar junction[18].

In a sexually mature woman, the cervical squamous epithelium is divided into 3 layers, basal/parabasal layer, midzone layer and superficial layer[19]. The cells of the basal layer have a scant cytoplasm and oval nuclei with dense chromatin[15]. The layer immediately above the basal layer has cells with increased cytoplasm and slightly less dense chromatin, called the parabasal layer[15]. The midzone layer is composed of intermediate cells with abundant cytoplasm and small vesicular nuclei[15]. These cells have glycogen accumulation due to estrogen stimulation giving a clear appearance to the cytoplasm[15]. The superficial cells exhibit a small, pyknotic, rounded nuclei with abundant cytoplasm and keratinization[15]. The keratinization is why the superficial and intermediate cells appear flat and platelike when spread on the slide[15].

The endocervix is lined by endocervical columnar cells with small basilar nuclei and cytoplasm containing mucin imparting a picket fence appearance to the epithelium[20]. Occasional ciliated cells and goblet cells may be found interspersed in the epithelium[15]. This endocervical epithelium dips into the stroma forming elongated clefts falsely termed as endocervical glands[21][20].

The endocervical mucosa is remains limited to the endocervical canal until puberty when due to hormonal stimulation the lips of the cervix swell and roll pulling the endocervical mucosa out of the canal into the exocervix[22]. This exposed

endocervical tissue is called the ectropion. As time goes by this endocervical tissue is replaced by squamous epithelium and the junction of these two epithelia is called squamo-columnar junction[22]. Two such junctions are recognized, the original junction and the functional squamo-columnar junction forming after active replacement of the endocervical epithelium[18]. The area between these two junctions is called the transformation zone which can be visualized on colposcopy[14].

The cervical stroma is composed of fibrous tissue with elastin and few strands of smooth muscle[23]. The stroma also contains a rich capillary network below the epithelium[23].

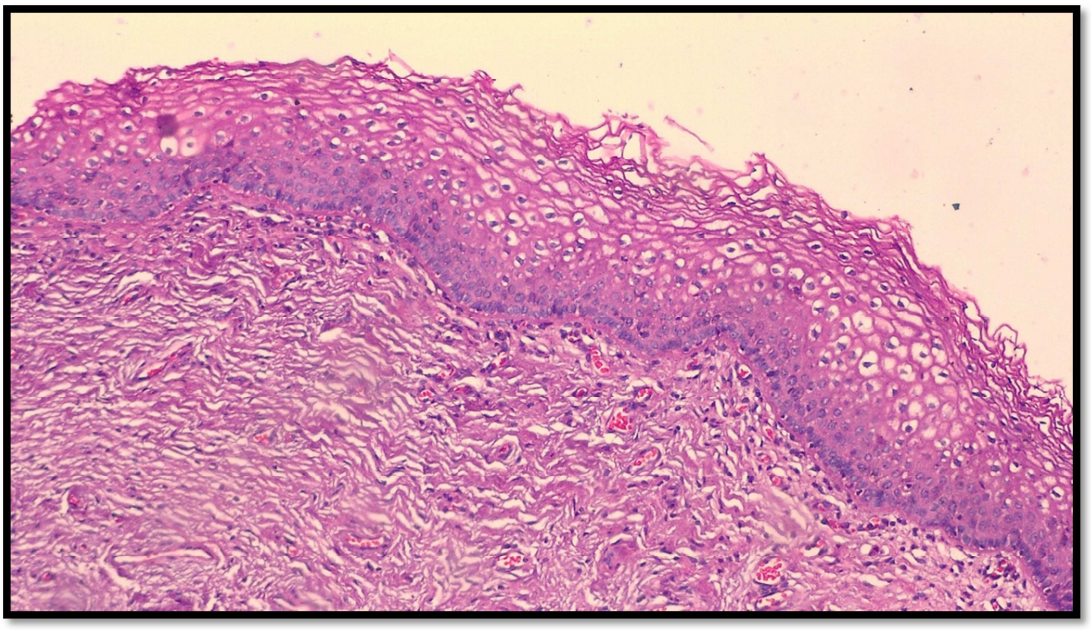


Figure 5 – Mature squamous epithelium of exocervix demonstration maturation sequence from basal to superficial cells.

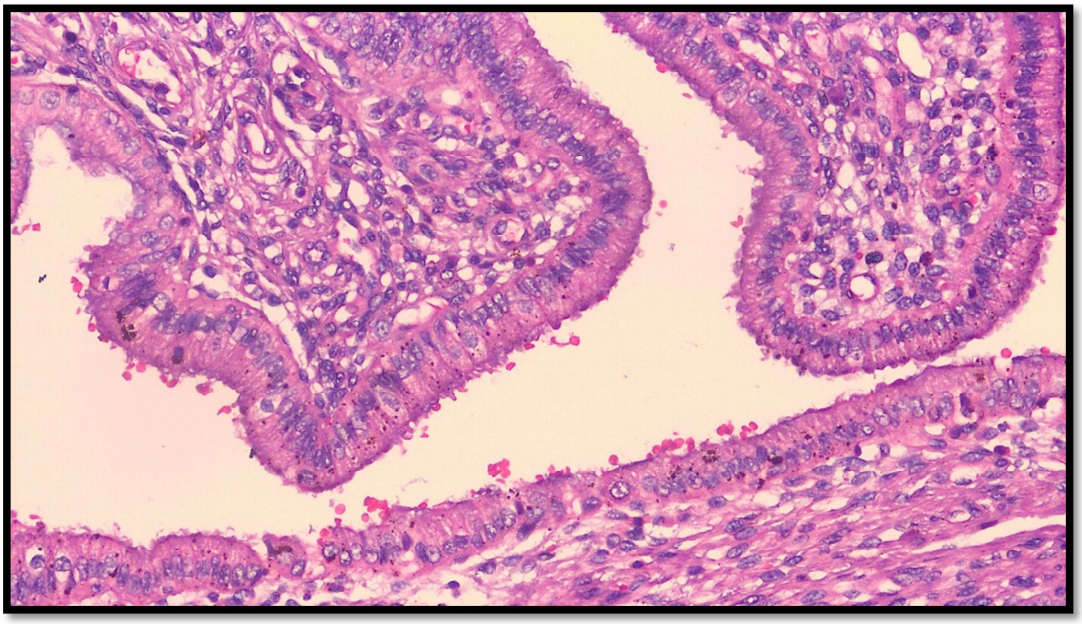


Figure 6 – Endocervical columnar epithelium showing nuclei in basilar location.

Physiology

The primary role of the cervix is production of cervical mucous which acts as a functional gate preventing the microorganisms in the vagina from entering the upper genital tract[24]. During the ovulatory period the mucous viscosity decreases allowing the passage of sperm and making the cervix environment more suitable for sperm viability[25]. The cervix also plays a vital role in supporting internal genitalia, labor and delivery[26].

History

The earliest evidence of writings about cervical carcinoma is from 400 BC by Hippocrates[27]. Aretaeus, a Greek physician in the 2nd century BC described all cancers of the uterus as presenting as ulcers that would infiltrate the uterus or growths in uterus[28]. Aretaeus also distinguished between these lesions based on symptoms and prognosis stating that lesions presenting as ulcers had a poor prognosis[28].

Rigonistern, a surgeon in the 19th century studied the pathogenesis of cervical carcinoma and created awareness about cervical carcinoma[28]. He studied the death certificates of women and observed that uterine cancer was rare in celibate nuns[28].

Epidemiologists in the 20th century observed the increased prevalence of cervical carcinoma in female sex workers and women with partners having multiple sexual partners[29]. Zur hausen and his colleagues identified HPV in 1983 in precancerous lesions. In 1985, HPV DNA was demonstrated in cervical cancer cells. This discovery eventually led to the research and development of HPV vaccinations[30]. The vaccinations approved against HPV by FDA are described in table 1[31].

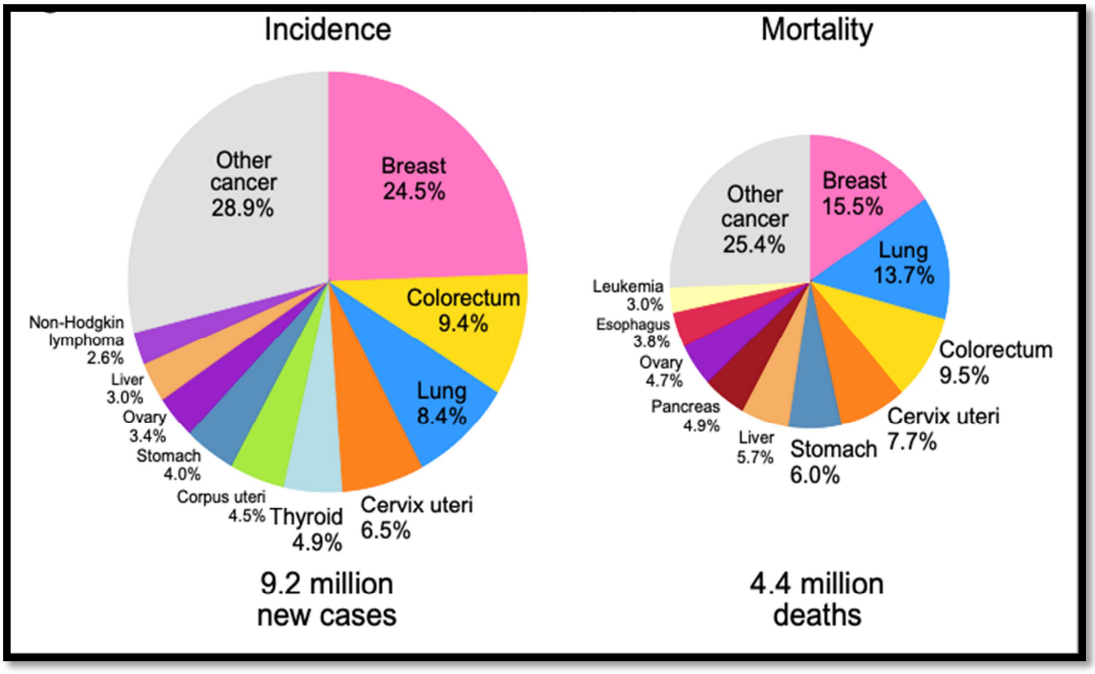
Cytologic screening has played a major role in reducing the morbidity and mortality of cervical carcinoma[28]. This is conducted using pap smears first discovered by George Papanicolaou in the year 1943 and published in the book named "Diagnosis of uterine cancer using vaginal smear"[32]. The reporting of pap smears was standardized by the Bethesda system in 1988 and further improved in 2001[28].

Burden of carcinoma cervix in the community

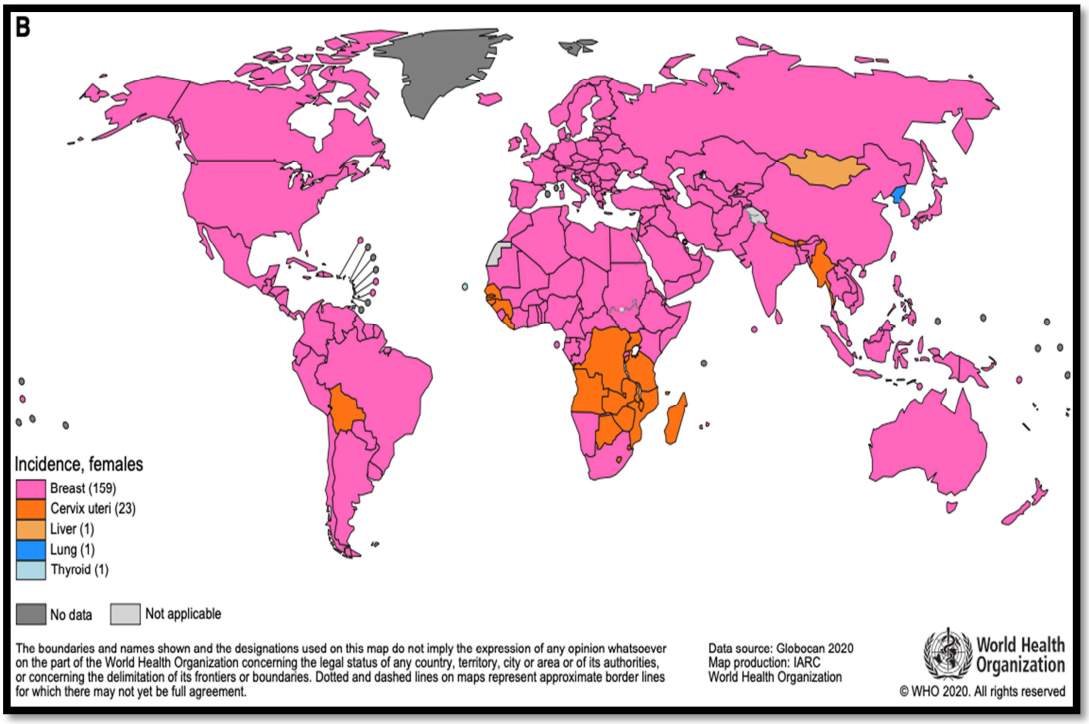
Globally cervical cancer accounted for 604,127 (3.1%) cases and 341,831 (3.4%) deaths in the year 2020[1]. It is the 4th most frequently diagnosed cancer and 4th leading cause of cancer mortality in women[1]. The rates of morbidity and mortality are high in developing vs developed countries and the disparity exists within high and low income areas in the United States[33]. This discrepancy between high and low income countries exists because of lack of a national HPV vaccination program and national cervical cancer screening program in developing nations[34].

It is the most commonly diagnosed cancer in 23 countries and leading cause of death in 36 countries[1]. HPV infection, HIV, Chlamydia trachomatis, smoking, high number of childbirths and long term use of contraceptives are factors involved in the carcinogenesis[1]. Along with breast cancer, cervical cancer accounts for the majority of global female cancer deaths[1].

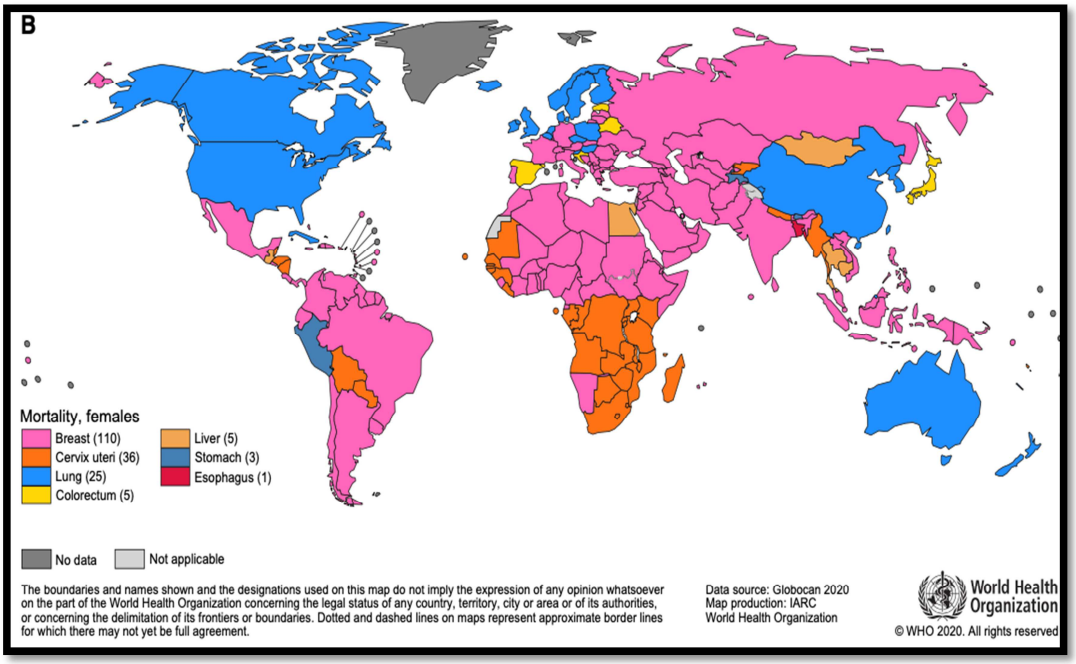
In India cervical cancer accounts for 6 to 29% of all cancer incidence in women and 17% of all cancer related deaths between the age of 30 and 69[35]. Studies conducted by Bobdey et al. provide evidence that visual inspection with acetic acid (VIA) and visual inspection with Lugol's iodine (VILA) are feasible and accurate screening tools[2].



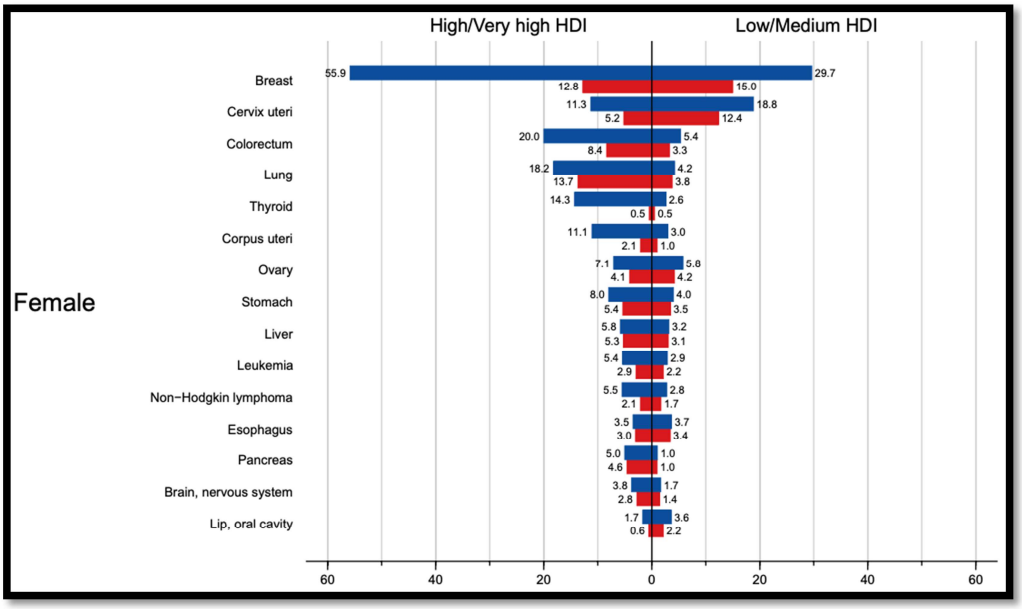
Graph 1– Distribution of incidence and mortality of the most common cancers in females in 2020[1].



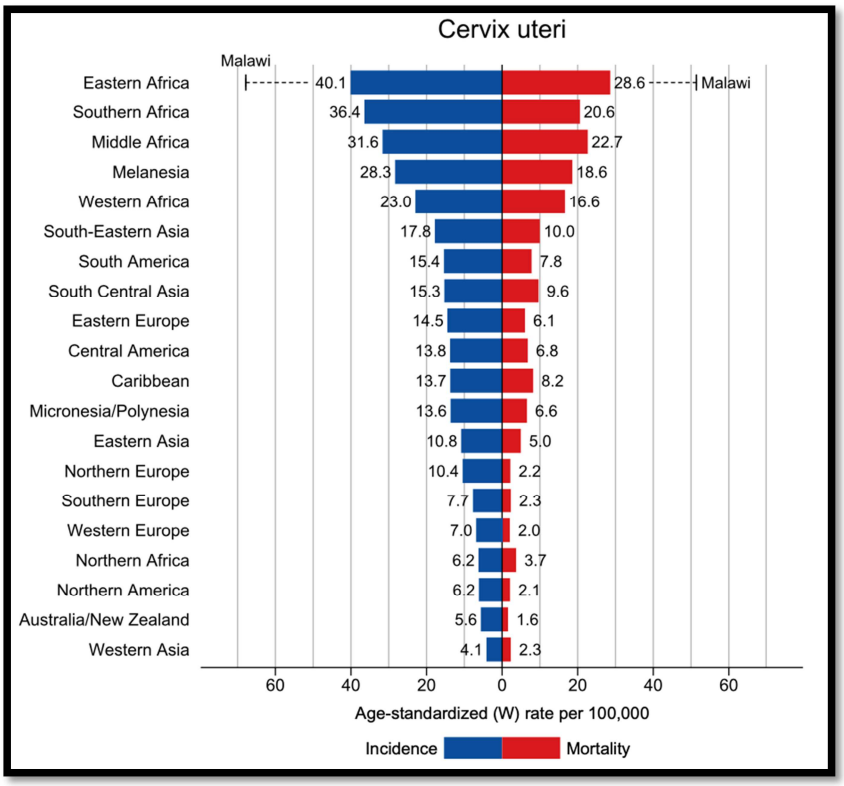
Graph 2- Most common type of cancer incidence in 2020 in each country among females[1].



Graph 3– Most common type of cancer mortality by country in 2020 among women[1].



Graph 4– Incidence and mortality age-standardized rates in High/very high human development index (HDI) countries and Low/ Medium HDI countries. The 15 most common cancers are arranged in descending order[1].



Graph 5 – Region specific incidence and mortality age- standardized rates for cervical cancer in 2020 in descending order[1].

Etiopathogenesis

The risk factors for carcinogenesis of cervix are low socioeconomic status, more than one sexual partner, first early intercourse (<16 years of age), increased parity, persistent high risk HPV infection, Immunocompromised, excess use of oral contraceptives, cigarette smoking, age – bimodal peak at 35 years and 55 years[36].

A normal cell cycle has G1 S and G2 M checkpoints to prevent unregulated cell proliferation[37]. Retinoblastoma protein and p53 protein play an important role in regulation of cell cycle[37]. In an HPV infected cell, oncoprotein E7 binds pRB preventing its association with E2F transcription factors resulting in its high free levels[37]. Oncoprotein E6 from was also shown to decrease intracellular levels of p53 which is responsible to prevent damaged DNA from replicating[37]. Thus, E6 and E7 oncoproteins from HPV together result in malignant transformation and propagation of DNA errors.

The incidence and mortality of cervical cancer has declined in many parts of the world because of increasing average socio-economic levels and diminishing risk of HPV infection due to improved genital hygiene and reducing incidence of sexually transmitted diseases[1]. HPV vaccination first began in 2006 as Gardasil which provided protection against HPV types 6, 11, 16, 18, 31, 33, 45, 52, 58[31].

The WHO currently recommends a intervention strategy of 1) 2-dose vaccination program for girls of 9 to 13 years[38], 2) screening program for women between 30 to 49 years via acetic acid visualization, Papanicolaou testing every 3 to 5 years or HPV testing every 5 years[38] and 3) treatment of precancerous lesions detected during testing[38].

Table 1 Prophylactic HPV vaccines approved by the FDA[31].

	Cervarix (2vHPV)	Gardasil (4vHPV)	GARDASIL 9 (9vHPV)
Manufacturer	GSK	Merck	Merck
Targeted HPV types	HPV16 and 18	HPV6, 11, 16, and 18	HPV6, 11, 16, 18, 31, 33, 45, 52, and 58
Recommended vaccination schedule	0, 1, and 6 months	0, 2, and 6 months	0, 2, and 6 months
Vaccine composition	20 mg HPV16 and 20 mg HPV18 VLPs	20 mg HPV6, 40 mg HPV11, 40 mg HPV16, and 20 mg HPV18 VLPs	30 μg HPV6, 40 μg HPV11, 60 μg HPV16, 40 μg HPV18, 20 μg HPV31, 20 μg HPV33, 20 μg HPV45, 20 μg HPV52, and 20 μg HPV58 VLPs
Recombinant protein expression system	Baculovirus (insect) cell	*Saccharomyces cerevisiae* (bread yeast)	*Saccharomyces cerevisiae* (bread yeast)
Adjuvant	500 mg aluminum hydroxide and 50 mg 3-*O*-desacyl-4′ monophosphoryl lipid A (MPL), a detoxified derivative of the lipopolysaccharide (LPS) of the Gram-negative bacterium *Salmonella* Minnesota R595 strain	225 mg amorphous aluminum hydroxyphosphate sulfate	225 mg amorphous aluminum hydroxyphosphate sulfate
FDA approvals (date) (link to package insert)	• 9- to 25-year-old females (16 October 2009) (http://www.fda.gov/downloads/BiologicsBloodVaccines/Vaccines/ApprovedProducts/UCM 186981.pdf)	• 9- to 26-year-old females (8 June 2006) • 9- to 26-year-old males (for genital warts) (16 October 2009) • 9- to 26-year-old males (for anal cancer) (22 December 2010) (http://www.fda.gov/downloads/BiologicsBloodVaccines/Vaccines/ApprovedProducts/UCM111263.pdf)	• 9- to 26-year-old females (10 December 2014) • 9- to 15-year-old males (10 December 2014) (http://www.fda.gov/downloads/BiologicsBloodVaccines/Vaccines/ApprovedProducts/UCM426457.pdf)
Current ACIP recommendations* [96]	• Females: Routine vaccination with three-dose series at age 11 or 12 years, and through age 26 years if not vaccinated previously	• Females: Age 11 or 12 years, and through age 26 years if not vaccinated previously • Males: Age 11 or 12 years, through age 21 years if not vaccinated previously, and through age 26 years for men who have sex with men and men who are immunocompromised (including those with HIV infection)	• Females: Age 11 or 12 years, and through age 26 years if not vaccinated previously • Males: Age 11 or 12 years, through age 21 years if not vaccinated previously, and through age 26 years for men who have sex with men and men who are immunocompromised (including those with HIV infection)

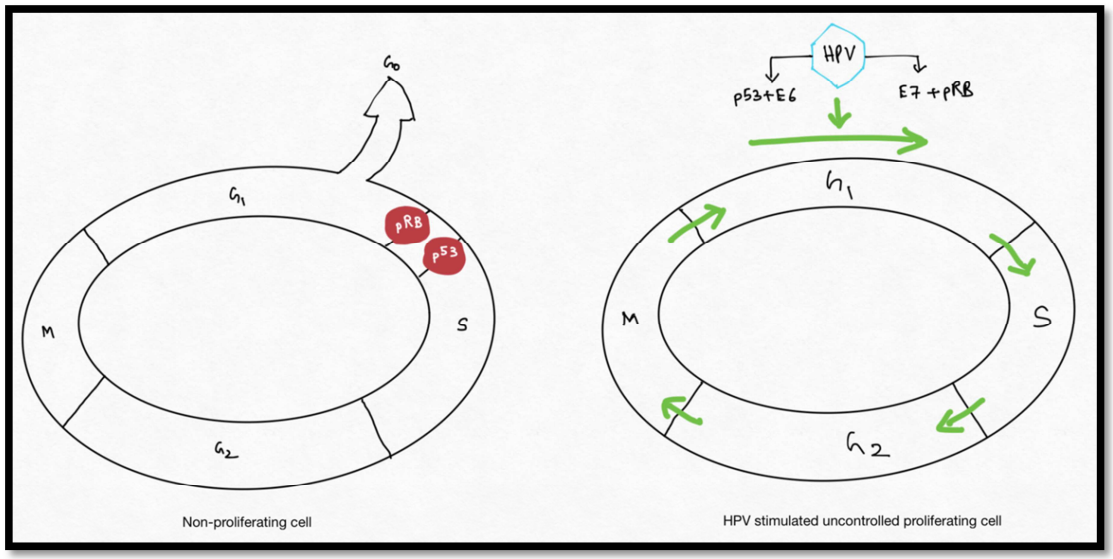


Figure 7 – Role of HPV oncoproteins in causing uncontrolled DNA replication[3].

SOX-2 Preamble

SOX2 proteins are transcription factors derived from the Sry gene located on the Y chromosome[39]. The HMG domain is part of the Sry gene which is similar to the HMG box superfamily[40]. The proteins grouped into the SOX protein family have more than 50% similarity to the HMG domain of Sry, also known as Sry box[41]. Thus, the name SOX 2 was derived[4]. The structure of this Sry-type HMG domain consists of 3 helices with the overall structure maintained by a hydrophobic core[42]. The SOX proteins bind to DNA on the minor groove allowing it to bind closely to other transcription factors and multiprotein complexes[43]. This binding also allows the proteins to link to other signal transduction pathways like WNT and TCF/LEF[43].

The primary function of SOX proteins is sex determination[44]. Sry protein located on Y chromosome, expressed in the genital ridge in embryo is a decisive factor for sex determination[44]. SOX2 protein plays an important role in embryogenesis, development of neural tissue and lens development[39]. SOX2 is associated with diseases like microphthalmia, syndromic 3 and septo-optic dysplasia[39].

SRR1 and SRR2 are the enhancers influencing the activity of SOX2 promoters, regulating SOX2 expression[45]. Micro-RNAs like miR-145 also play a important regulatory role for SOX2 expression and are implicated in many of the cancers[46]. Other microRNAs like miR-126 may play a role in tumor suppression in hepatocellular and osteosarcoma[47]. SOX2 levels appear to be optimized for maximum tumor growth as it is inhibitory when its levels are too high or low[9].

The concept of cancer stem cell (CSC) hypothesis suggests that tumour masses arise from a single cancer cell with stem cell like properties[48]. In cervical cancer, SOX2 is considered to be one of the potential markers for CSCs[49]. This is because SOX2 is identified to be expressed as an oncogene in primary cervical cancer tissues controlling pluripotency, self-renewal, proliferation of embryonic stem cells[50]. This suggests that SOX2 is a nuclear marker for CSCs[51]. Study conducted by Xiao-Fang Liu et. al. isolated SOX2 positive cancer cells using a pSOX2/EGFP plasmid and found these cells to have all the properties of CSCs[52]. The cancer stem cells play a crucial role in tumour initiation and aggressiveness via mechanisms like lineage plasticity, immune surveillance, anti-apoptotic signalling, epithelial to mesenchymal transition[9]. Thus, SOX2 can be considered in future as a therapeutic target for CSC specific anti-cancer therapies[51].

SOX2 plays its role of tumorigenesis via various modifications at transcriptional level mediated by signal transduction and translational level by microRNA and post translational level by phosphorylation, methylation and acetylation[5].

The SOX family comprises of various transcription factors and their clinical significance is as follows. SOX1 is a transcription factor with anti-tumour activity[5]. It is downregulated by miRNA-155 increasing metastasis in gastric carcinoma while inhibition of Wnt/beta-catenin pathway results in overexpression of SOX1 and impaired metastasis in cervix and breast[5]. SOX2 in correlation with OCT-4 and signaling pathways like WNT, FGF is shown to play a role in give breast carcinomas a basal cell/stem cell like phenotype with pluripotency and self-renewal[6].

SOX2 is not only expressed in cervical squamous cell carcinomas but also plays a role in other carcinomas like breast, gastric, pancreas, lung. In the human digestive tract SOX2 expression is normally found in the stomach but not the intestine[53]. Otsubo et. al found that expression of SOX2 is significantly downregulated in gastric carcinomas as it acts as a tumor suppressor[53]. SOX2 is known to colocalize with p53 in the basal layer and plays a crucial role in maintaining the stratified squamous epithelium of the git[54]. It was found that SOX2 was preferentially expressed in esophageal and anal canal squamous cell carcinomas compared to adenocarcinomas[54]. Sholl et. al demonstrated that SOX2, a marker of embryonic cell pluripotency is associated with aggressive tumor behavior in stage 1 lung adenocarcinomas[7]. Rodriguez-Pinilla el. al observed that SOX2 expression was significantly more in basal like breast carcinomas compared to other subtypes of breast carcinomas[6]. An inverse association between SOX2 and ER, PR was also identified indicating that SOX2 plays an essential role in maintaining stem cell like phenotype of breast carcinomas[6]. Sanada et. al observed that SOX2 plays a role in the carcinogenesis, invasion and metastasis of pancreatic intraepithelial neoplasia[55]. SOX2 gene is also known to be hypermethylated resulting in reduced RNA expression in hydatidiform moles compared to normal placentas[56].

In the cervix, SOX2 was found to be expressed in 20% of normal cervix and 75% of cases with cervical squamous cell carcinoma[52]. No correlation was found between the grade or stage of tumor and SOX2 expression[52]. It is speculated that this increase in SOX2 expression is due to the presence of cancer stem cells[57]. SOX2 is expressed in undifferentiated embryonic stem cells suggesting they play an important role in maintaining the pluripotency and self-renewal of tumor cells[58].

Moshi et. al studied the expression of SOX 2 and SOX 17 in the transformation zone of the cervix[59]. They found that SOX 2 is exclusively found in the squamous epithelium and SOX 17 is found in the endocervical columnar epithelium[59]. As the transformation zone undergoes squamous metaplasia it loses SOX 17 expression and gains SOX 2 expression[59]. The reserve cells in the epithelium were also found to express SOX 17[59]. On being infected with HPV it was found that the epithelium changes SOX 17 to SOX 2 expression[59].

The Wnt pathway is known to play a significant role in the regulation of stem cells via beta catenin gene expression[60]. Beta catenin is known to play a significant role in tumorigenesis of colorectal carcinoma[60]. Ji et. al. in their study found that there is a significant association between SOX2 and beta catenin expression in cervical squamous cell carcinoma indicating its role in tumorigenesis[60]. The same study also found a significant association between SOX2 and survival of squamous cell carcinoma patients[60]. Another marker for cancer stem cells is FOXP3 and study conducted by Kldiashvili et. al. showed an association between FOXP3 and SOX2 expression[61].

The hedgehog pathway is another pathway known to play a significant role in tumorigenesis of cervical squamous cell carcinoma via Gli1[62]. Gli1 is a transcriptional factor responsible for the effector action of hedgehog pathway and the expression levels are known to be associated with SOX2[63]. Huang et. al proved a role of SOX2 in radio-resistance resulting in treatment failure and recurrence[62]. SOX2 is proposed as a potential therapeutic target[63].

P16 protein is a tumor suppressor protein preventing cell proliferation and its expression is reduced in HPV infection[64]. Dayalan et. al. found a correlation between SOX 2 and p16 expression[64]. Another study found reduced expression of SOX2 and TP53 in HPV infected cervical squamous cell carcinoma[65]. This indicates SOX2 plays a significant role in tumorigenesis of HPV induced cervical squamous cell carcinoma[64].

As SOX2 is a stem cell marker and responsible for stem cell renewal and pluripotency it is associated with poor prognosis in various cancers[66]. In cervical squamous cell carcinomas it was correlated with advanced tumor size, tumor grade and cancer progression[67]. Thus, SOX2 can serve as a potential therapeutic target[67].

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Table 2– Classification and functions of SOX proteins[39]

Group	Factor	Expression pattern	Function
A	SRY	Male embryonic genital ridge	Testis determination
B	Sox1	Embryonic central nervous system	Lens development
	Sox2	Primitive ectoderm, lens, developing CNS	Activation of FGF-4, repression of osteopontin gene
	Sox3	Embryonic CNS, urogenital ridge	X-linked mental retardation syndromes
	Sox19	Presumptive CNS in early gastrula, diencephalon, midbrain, hindbrain	
	Sox21	Primitive ectoderm, endoderm, embryonic CNS	
	Sox70	Trunk of syncytial blastoderm, ventral and cephalic neuroectoderm	Regulation of pair rule genes
C	Sox21	Embryonic heart, developing CNS	Endocardial ridge development, B-cell development
	Sox11	Developing CNS, PNS, facial mesenchyme, limbs, lung, kidney	Neural determination and differentiation events
D	Sox22	Throughout CNS, PNS, kidney, pancreas	Neural determination and differentiation events
	Sox24	Oocytes	
E	Sox5	Adult testis	Spermatogenesis
	Sox6	Embryonic CNS, mesenchymal condensations	Chondrogenesis
	Sox12	Ovary	
	Sox13	Embryonic ear, thymus, ovary	

	Sox23	Brain, ovary, liver	
F	SoxP1	Pituitary, gonads	
	Sox9	Mesenchymal condensations	Chondrogenesis, sex determination
	Sox10	Neural crest, embryonic PNS, CNS	Neural crest cell determination
G	Sox7	Gonads, kidney, lung, brain	
	Sox17	Testis	
	Sox18	Lung, cardiac, skeletal muscle	
H	Sox20	Fetal testis	

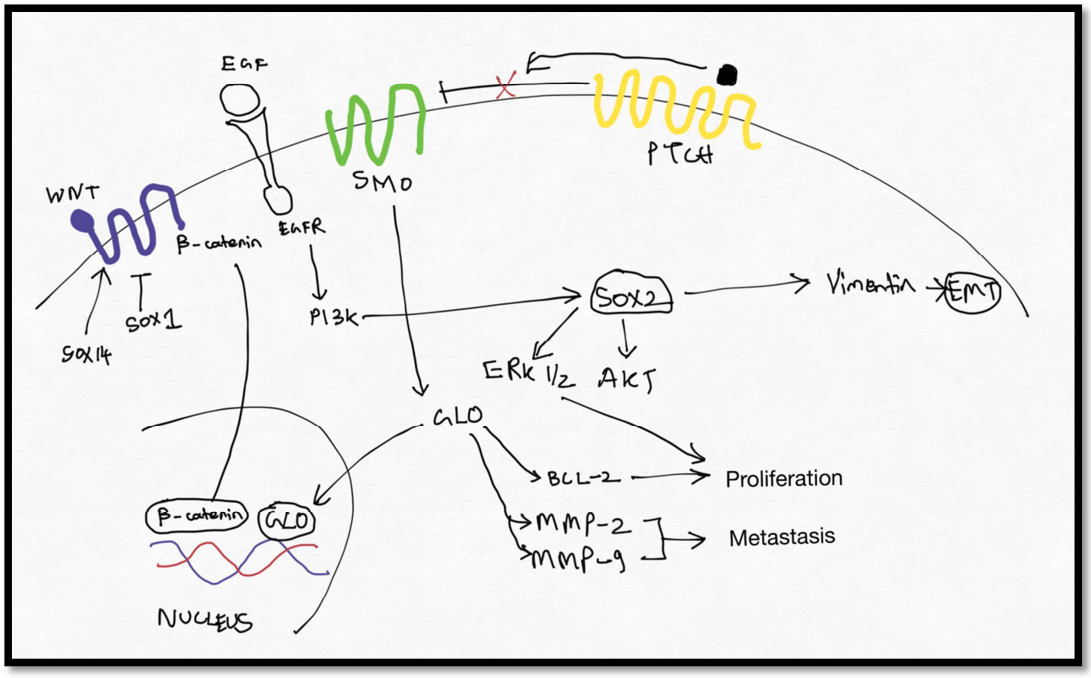


Figure 8– Role of SOX1, SOX2, SOX14 in cancer related molecular pathways[68].

METHODOLOGY

The study was carried out at the Department of Pathology, KAHER's Jawaharlal Nehru Medical College, and Dr. Prabhakar Kore charitable Hospital and medical research centre, Belagavi. Patient identities were concealed and approval from the KAHER University's Institutional Research Ethical Committee was obtained prior to conducting this study.

Study design: Cross sectional study

Period: One year prospective (1st January 2021 to 31st December 2021) and three years retrospective cross-sectional study.

Study population: All cases of cervical squamous cell carcinoma [biopsy and hysterectomy specimen] received in the histopathology laboratory of the Department of Pathology, JN Medical college and Dr. Prabhakar Kore Hospital and MRC. For retrospective cases, data as well as tissue blocks will be retrieved from from the medical records centre & department archives.

Inclusion criteria: All samples of- Cervical Squamous cell carcinomas. Including all variants- keratinising, non- keratinising, basaloid, verrucous, warty, papillary, squamo-transitional etc.

Exclusion criteria: All non-squamous carcinomas like -Metastatic lesions of cervix, Adenocarcinoma of cervix, Cervical intraepithelial neoplasia.

Sample size: 50. Within past 3 years 13,12, 11 samples were collected respectively from KLE'S DR. PRABHAKAR KORE HOSPITAL, BELAGAVI. This amounts on

average of approximately 10 to 15 samples in a year. Thus, all the cases in the study period are included using universal sampling method.

Data collection: Preparation of slides from tissue blocks: Approximately 35 – 40 cases received in department of pathology, JNMC and KLE Prabhakar Kore hospital in Belagavi will be retrieved and H&E slides will be prepared.

Grading of slides: H&E slides will be graded according to Broder's histopathological grading to determine degree of differentiation. The tumours will be scored as high, moderate or poor differentiation.

Immunohistochemistry staining: The slides will be stained with SOX-2 antibody. IHC slides will be evaluated for pattern, intensity and percentage of positivity of neoplastic cells. The nature of the background non-neoplastic cells will be noted. Each case will be assigned a score based on degree of expression.

Correlation and Data Analysis: The degree of differentiation score determined from histopathological grading will be compared with the intensity score of SOX-2 expression. Data will be coded and entered in Microsoft Excel and analysed in statistical package for social sciences - version 19. Comparative analysis will be done using descriptive statistics and chi-square test. The p-value of 0.05 or less will be considered statistically significant.

Sampling procedure: Universal Sampling

H and E analysis: 50 formalin fixed paraffin embedded blocks of cervical biopsies diagnosed as cervical squamous cell carcinoma were collected from departmental records. 3 micron thick ribbons were obtained using rotary microtome and floated in

tissue floatation bath maintained at 50-55 degree Celsius. One tissue ribbon was lifted on a regular glass slide and stained with hematoxylin and eosin. The H and E slides were evaluated as per the following table. Other ribbon was taken on Poly – L – Lysine coated glass slides for immunohistochemistry analysis.

Table 3 – Broder's grade in squamous carcinomas(69).

Broder's grade	Score
Well differentiated	1
Moderately differentiated	2
Poorly differentiated	3

IHC analysis: Only nuclear or nuclear with cytoplasmic staining was considered positive. Scoring for SOX2 was done as(70,71)-

Table 4 – Percentage of positive cells.

Percentage of positive cells	Score
<10 %	0
10 – 25 %	1
25 – 50 %	2
51 – 75 %	3
>75%	4

Table 5 – Intensity of positive staining.

Intensity	Score
Negative	0
Weak	1
Medium	2
Strong	3

Table 6 – IHC score calculation and interpretation.

IHC score (Positive cell % X Intensity)	Interpretation
0	Negative
1-4	Weak positive
5-8	Positive
9-12	Strong positive

Statistics: The degree of differentiation score determined from histopathological grading was compared with the intensity score of SOX-2 expression. Data was coded and entered in Microsoft Excel and analysed in statistical package for social sciences - trial 19. Comparative analysis will be done using descriptive statistics and Spearman's rank correlation test. The p-value of 0.05 or less will be considered statistically significant.

RESULTS

In the present study conducted in the Department of pathology, Jawaharlal Nehru Medical College, Belagavi, 50 cases of cervical biopsy specimens which were histopathologically diagnosed as SCC cervix were evaluated for SOX2 expression.

Table 7 – Age distribution of the cases.

AGE	NUMBER	%
35- 44	8	16.00
45 -54	15	30.00
55 - 64	21	42.00
65 - 74	6	12.00
TOTAL	50	100.00

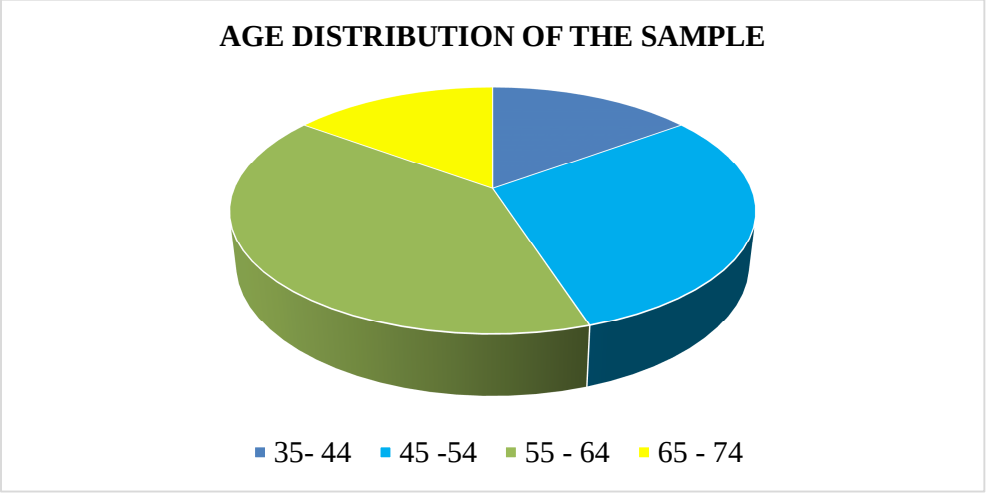


Figure 6 – Pie chart of age distribution of the cases.

The study comprised of patients aging 35-74 years. The mean and median age was 53.44 years and 55 years respectively. The peak incidence was between 55-64 years comprising 42% of the cases. (**Table 1, Figure 15**)

Table 8 – Case specimen type.

SPECIMEN TYPE	NUMBER	%
ENDOCERVICAL CURETTAGE	1	2.50
HYSTERECTOMY	3	7.50
PUNCH BIOPSY	46	90.00
TOTAL	50	100.00

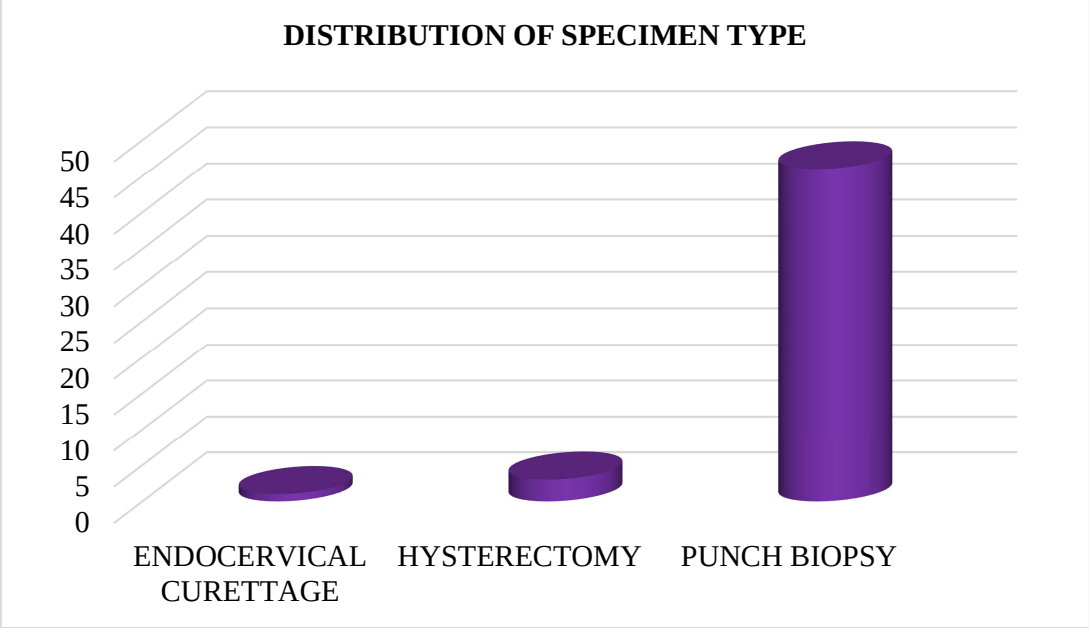


Figure 7 – Graph of distribution of specimen type.

90% of the cases in this study were punch biopsies, 3 hysterectomy and 1 endocervical curettage specimens were also included. (**Table 2, Figure 16**)

Table 9 – Distribution of Broder's grade.

BRODER'S GRADE	NUMBER	%
1	6	12.00
2	38	76.00
3	6	12.00
TOTAL	50	100.00

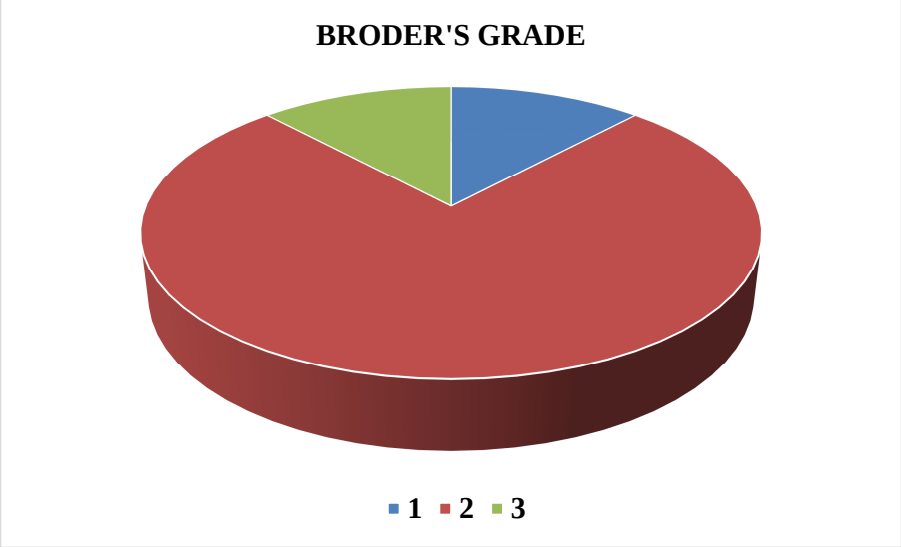


Figure 8 – Pie chart of Broder's grading distribution.

On hematoxylin and eosin it was found that 76.00% or 38 of the cases were moderately differentiated squamous cell carcinoma, 12.00% were poorly differentiated and 12% were well differentiated. (**Table 3, Figure 17**)

Table 10 – SOX2 percentage positive cells and Broder's grade.

BRODER'S GRADE	PERCENTAGE OF POSITIVE CELLS					TOTAL	P Value
	<10 %	**10 – 25 %**	**25 – 50 %**	**51 – 75 %**	**>75%**		
Well	0 (0%)	2(4%)	4 (8%)	1 (2%)	0 (0%)	7 (14%)	0.3131 (NS)
Moderate	3 (6%)	09 (18%)	12 (24%)	10 (20%)	3 (6%)	37 (74%)	
Poor	0(0%)	1(2%)	2 (4%)	1 (2%)	2(4%)	6 (12%)	
Total	3 (6%)	12 (24%)	18 (36%)	12 (24%)	5 (10%)	50(100%)	

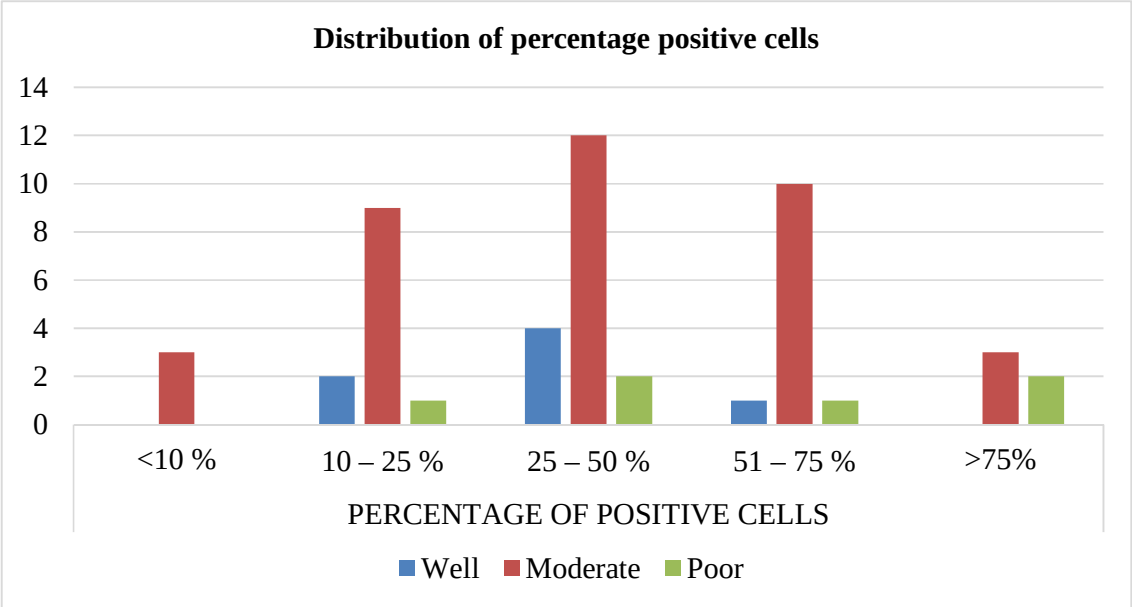


Figure 9 – Distribution of SOX2 percentage positive cells.

After SOX2 staining it was found that the majority cases showed 25-50% of neoplastic cells expressing SOX2 and another 12 cases showed 51-75% SOX2 expression. Only 5 cases showed more than 75% of cells expressing SOX2 and 3 cases showed less than 10% cells expressing SOX2. Well differentiated carcinoma showed 25-50 % cells expressing SOX2 while moderately differentiated carcinoma also had majority of cases with 25-50% cells expressing SOX2. (**Table 4, Figure 18**)

Table 11- Intensity of SOX2 staining.

BRODER'S GRADE	INTENSITY				TOTAL	P Value
	NEGATIVE	WEAK	MEDIUM	STRONG		
Well	0 (0%)	0 (0%)	2 (4%)	4 (8%)	6 (12%)	0.8194 (NS)
Moderate	2 (4%)	1 (2%)	9 (18%)	26 (52%)	38 (76%)	
Poor	0 (0%)	0 (0%)	4 (8%)	2 (4%)	6 (12%)	
Total	2 (4%)	1 (2%)	15 (30%)	32 (64%)	50 (100%)	

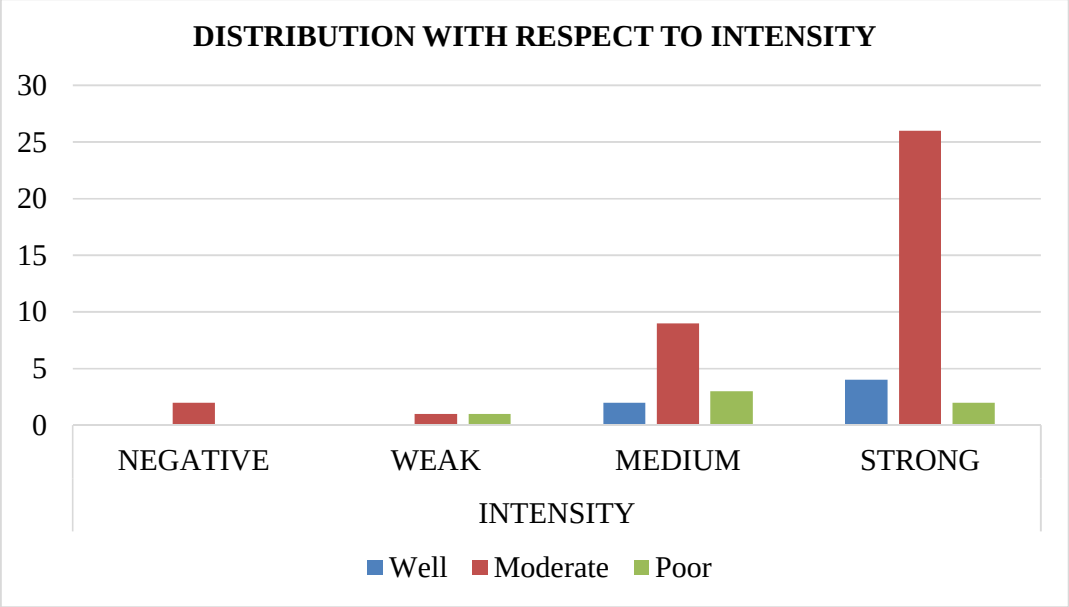


Figure 10 – Distribution of the intensity of SOX2 expression.

A majority of the cases showed strong intensity of SOX2 expression. Two of the cases were negative with no nuclear staining both of them being moderately differentiated on H and E. One of the cases with moderately differentiated squamous cell carcinoma had weak SOX2 expression. There was positive correlation found with the intensity of SOX2 expression in both well and moderately differentiated squamous cell carcinomas. (**Table 5, Figure 19**)

Table 12– IHC expression score.

BRODER'S GRADE	HISTOLOGIC SCORE (POSITIVE CELL % X INTENSITY)				TOTAL	P Value
	NEGATIVE	WEAK	MEDIUM	STRONG		
Well	0(0%)	3(6%)	2(4%)	1(2%)	6 (12%)	0.9961 (NS)
Moderate	2(4%)	15(30%)	11(22%)	10(20%)	38 (76%)	
Poor	0(0%)	3(6%)	1(2%)	2(4%)	6 (12%)	
Total	2(4%)	21(42%)	14(28%)	13(26%)	50 (100%)	

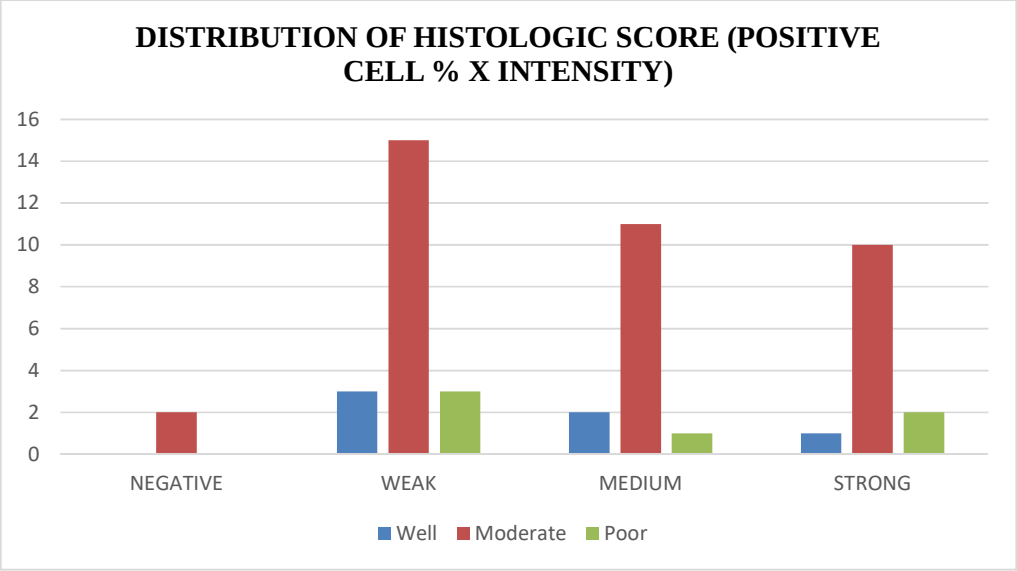


Figure 11 – The distribution of IHC expression score for SOX2.

The overall IHC expression score was predominantly weak seen in 42% of the cases. The expression was medium and strong in 28 and 26% of the cases respectively. SOX2 expression was predominantly weak in moderately differentiated squamous cell carcinomas. (**Table 6, Figure 20**)

Table 13 – Spearman rank correlation coefficients for well differentiated squamous cell carcinomas.

PARAMETERS FOR WELL DIFFERENTIATED	CORELATION COEFFICIENT	p VALUE	INFERENCE
BETWEEN AGE AND IHC SCORE	0.3875	0.4542	NS
BETWEEN BRODER'S GRADE AND PERCENTAGE OF POSITIVE CELLS	0.2375	0.1237	NS
BETWEEN BRODER'S GRADE AND INTENSITY SCORE	0.0459	0.6453	NS
BETWEEN BRODER'S GRADE AND IHC SCORE	0.1312	0.2102	NS

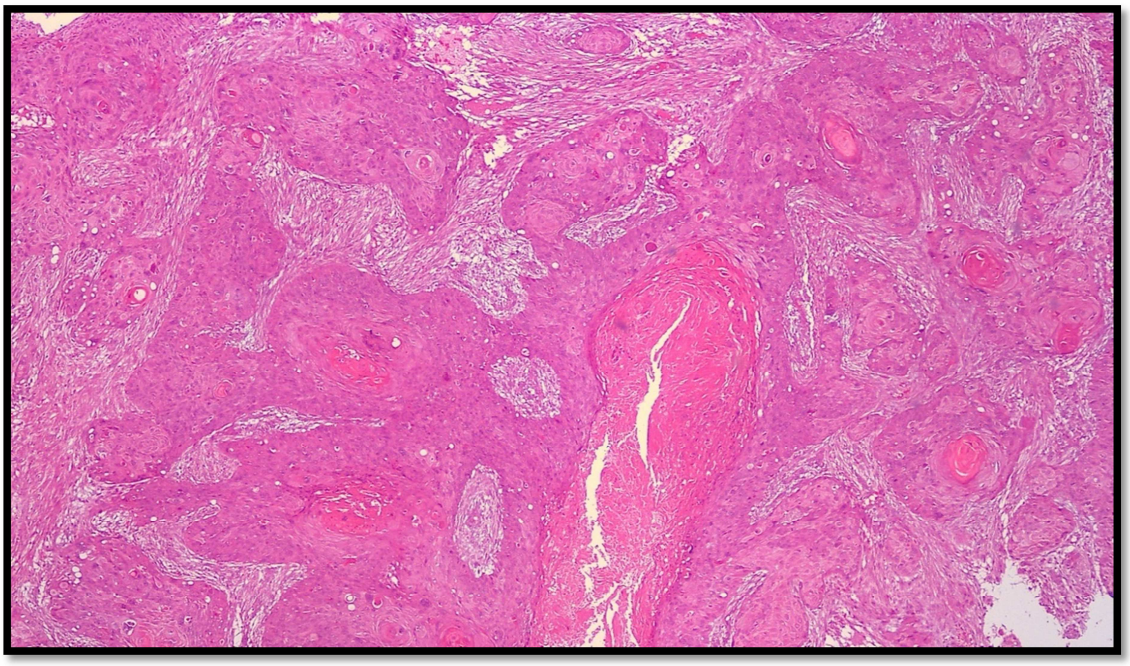
Table 14 – Spearman rank correlation coefficients for moderately differentiated squamous cell carcinomas.

PARAMETERS FOR MODERATELY DIFFERENTIATED	CORELATION COEFFICIENT	p VALUE	INFERENCE
BETWEEN AGE AND IHC SCORE	0.1861	0.2632	NS
BETWEEN BRODER'S GRADE AND PERCENTAGE OF POSITIVE CELLS	0.3145	0.3409	NS
BETWEEN BRODER'S GRADE AND INTENSITY SCORE	0.1492	0.4532	NS
BETWEEN BRODER'S GRADE AND IHC SCORE	0.2731	0.1675	NS

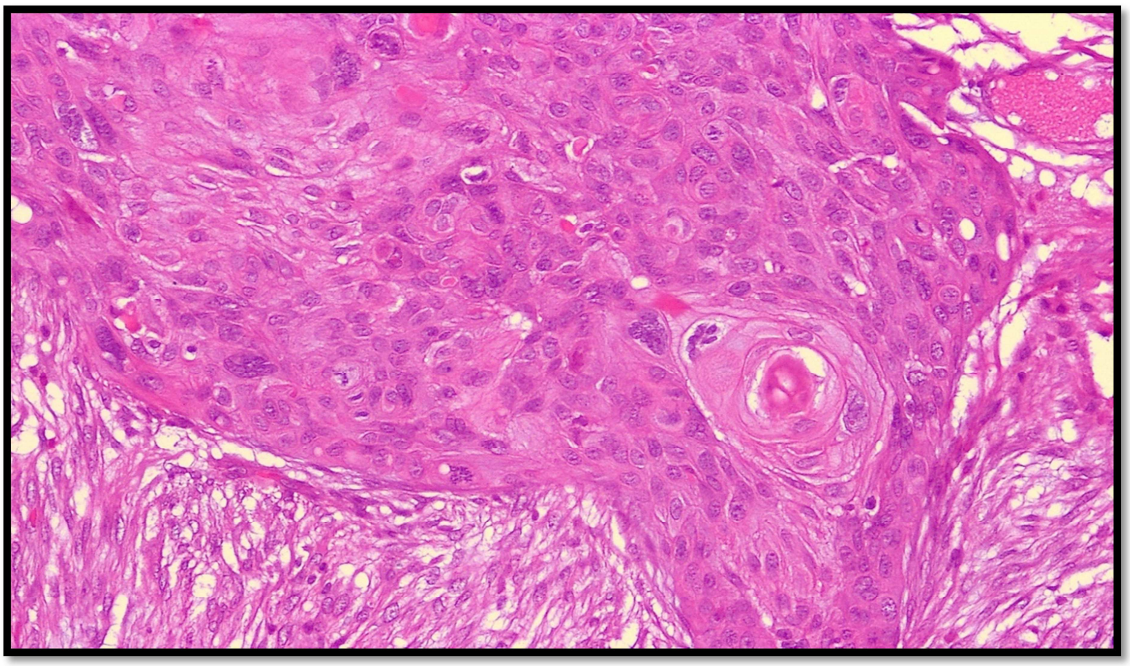
Table 15 – Spearman rank correlation coefficients for poorly differentiated squamous cell carcinomas.

PARAMETERS FOR POORLY DIFFERENTIATED	CORELATION COEFFICIENT	p VALUE	INFERENCE
BETWEEN AGE AND IHC SCORE	0.7062	0.1168	NS
BETWEEN BRODER'S GRADE AND PERCENTAGE OF POSITIVE CELLS	0.4213	0.4932	NS
BETWEEN BRODER'S GRADE AND INTENSITY SCORE	0.1219	0.5843	NS
BETWEEN BRODER'S GRADE AND IHC SCORE	0.3265	0.2476	NS

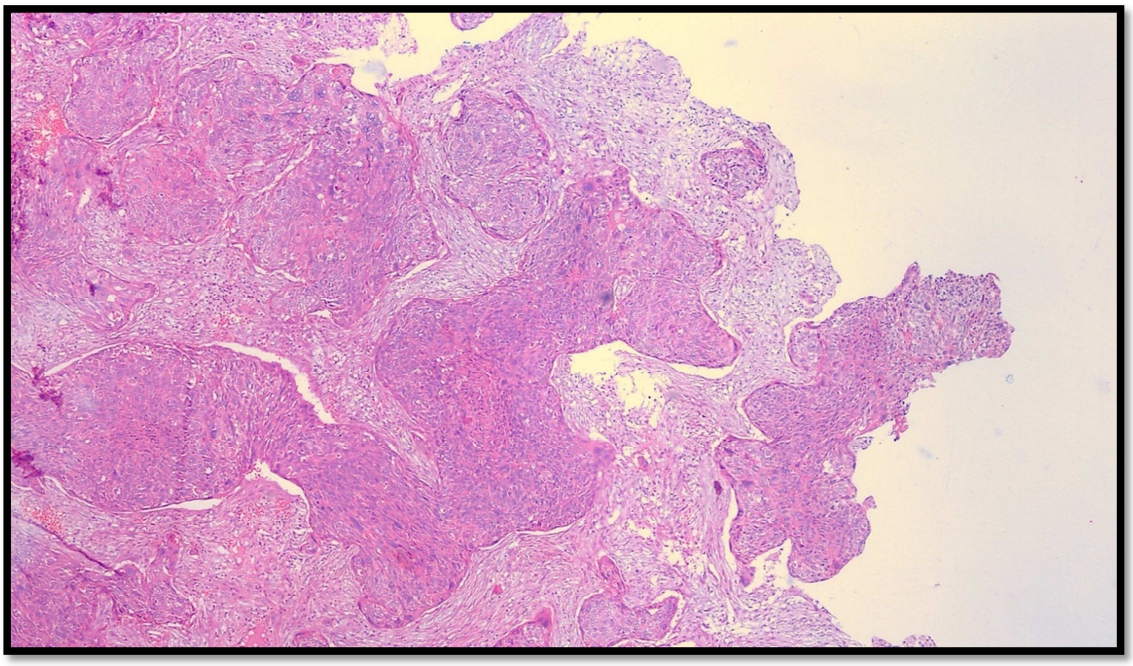
There was no significant correlation found between IHC score and age or between Broder's grade and percentage of positive cells or intensity. No correlation was found between Broder's grade and IHC even when each grade group was evaluated individually. (**Table 7, 8, 9**).



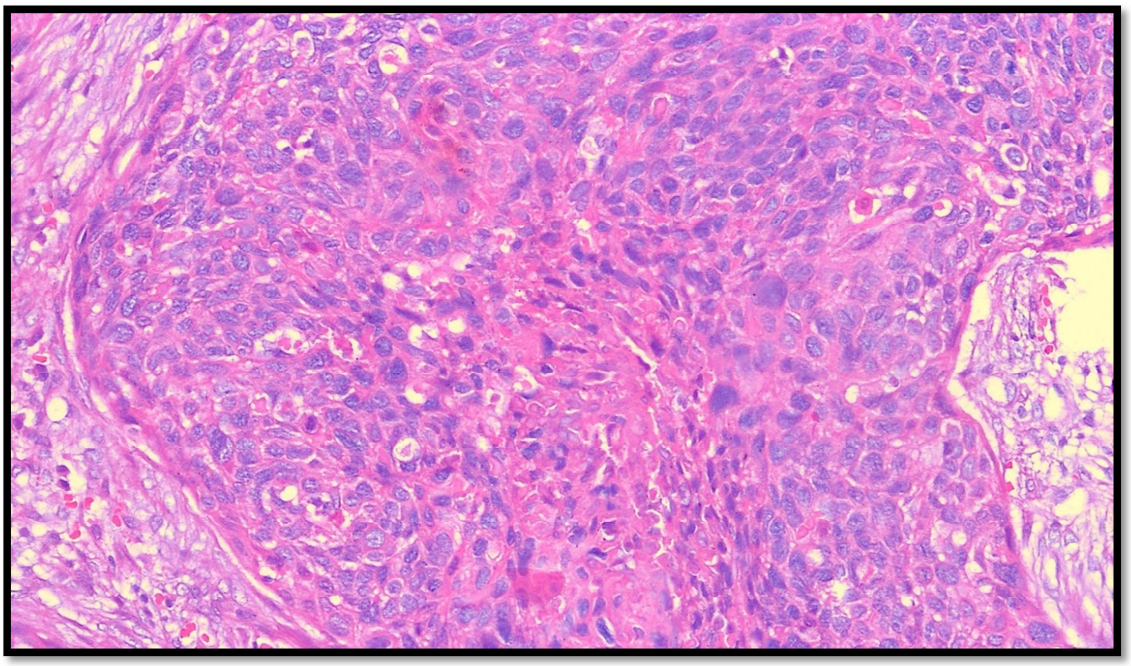
Photomicrograph 1: Well differentiated SCC with keratin pearls (H&E, 40x)



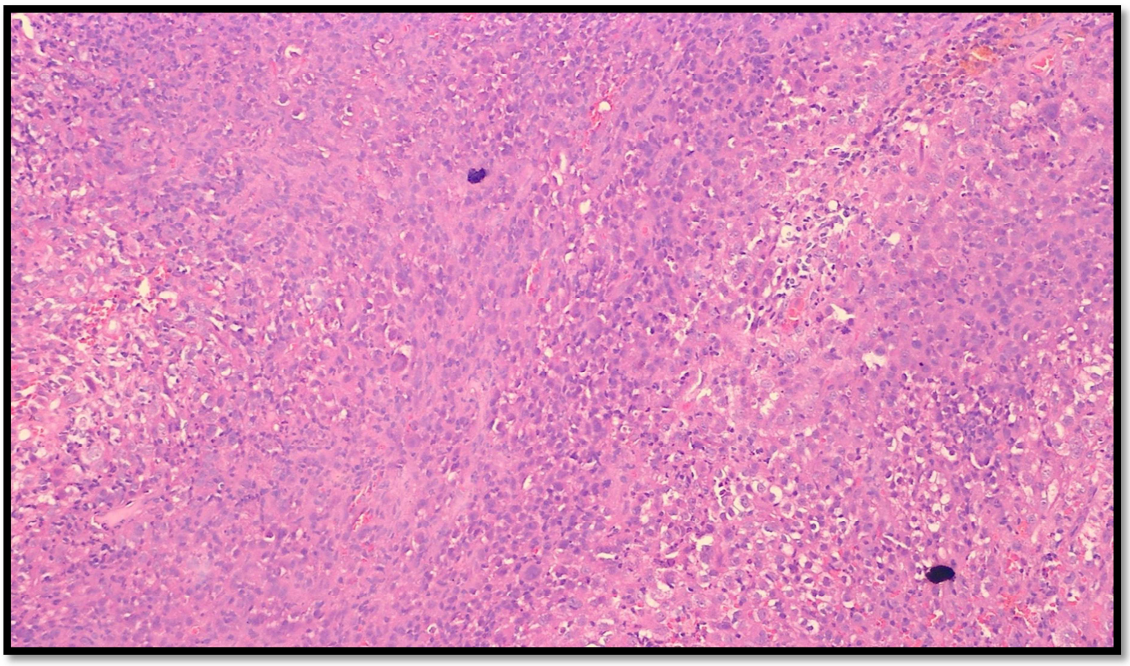
Pictomicrograph 2 - Well differentiated SCC with keratin pearls (H&E, 400x)



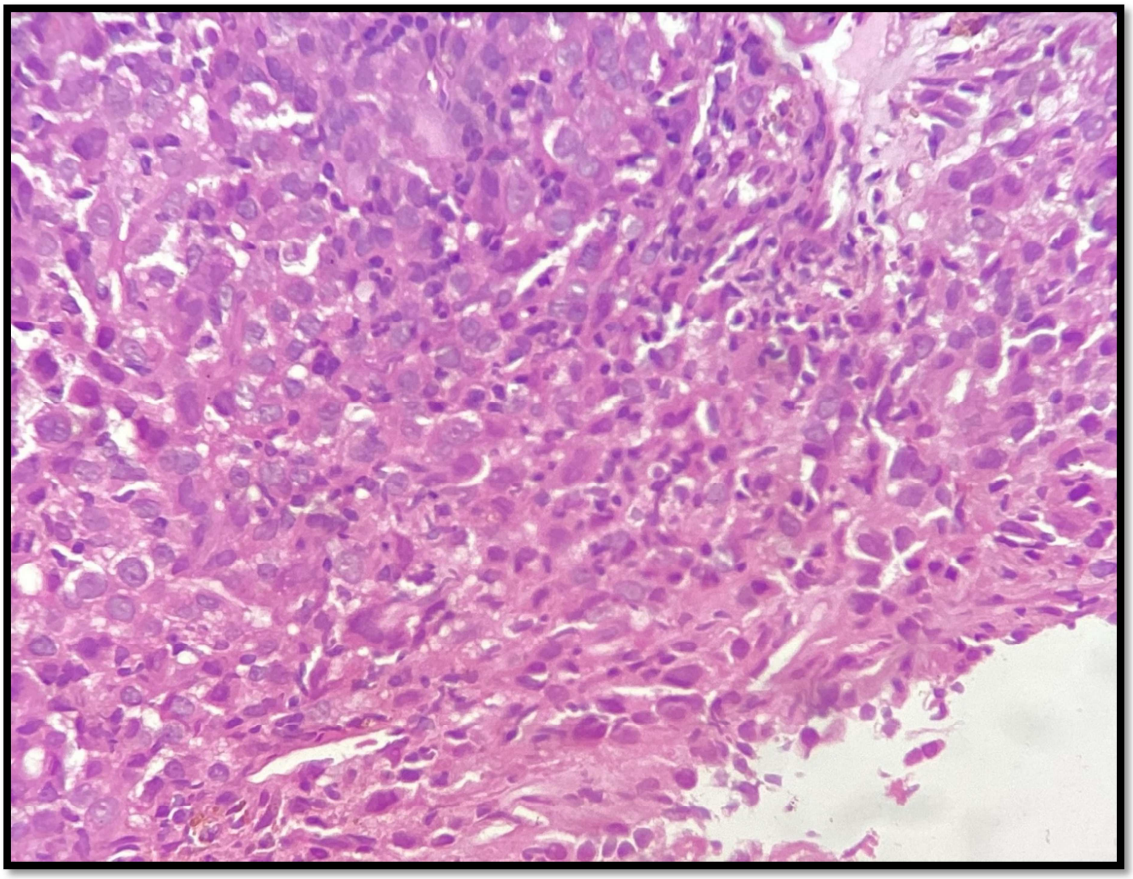
Pictomicrograph 3 – Moderately differentiated SCC (H&E, 40x)



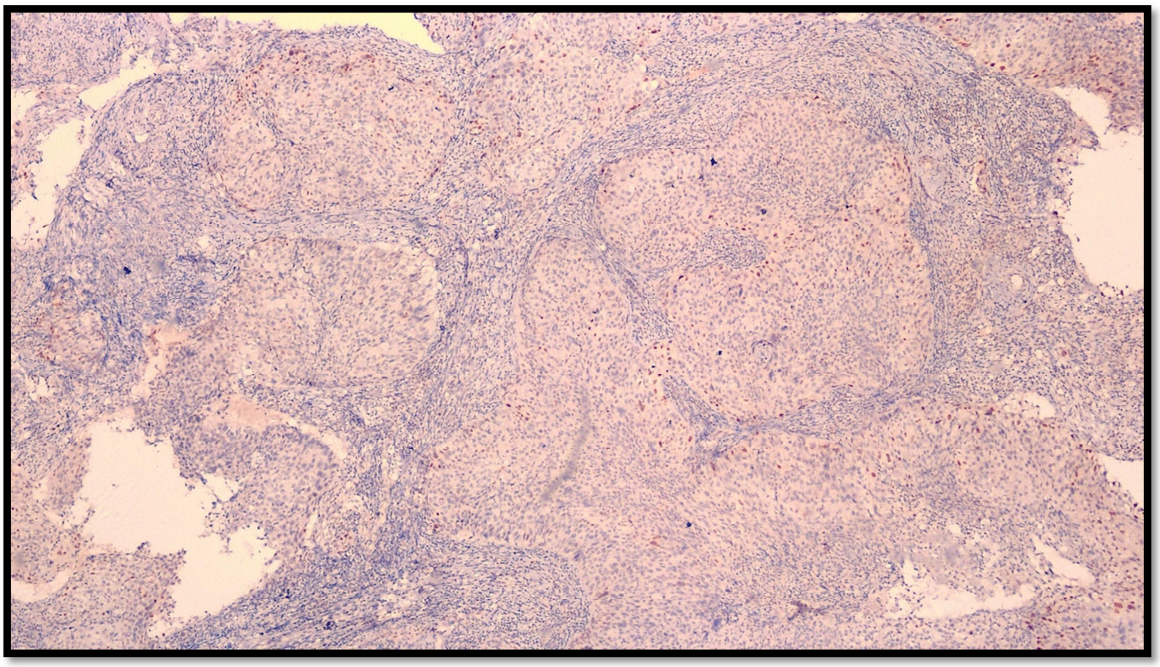
Pictomicrograph 4 – Moderately differentiated SCC (H&E, 400x)



Pictomicrograph 5 – Poorly differentiated SCC (H&E, 40x)

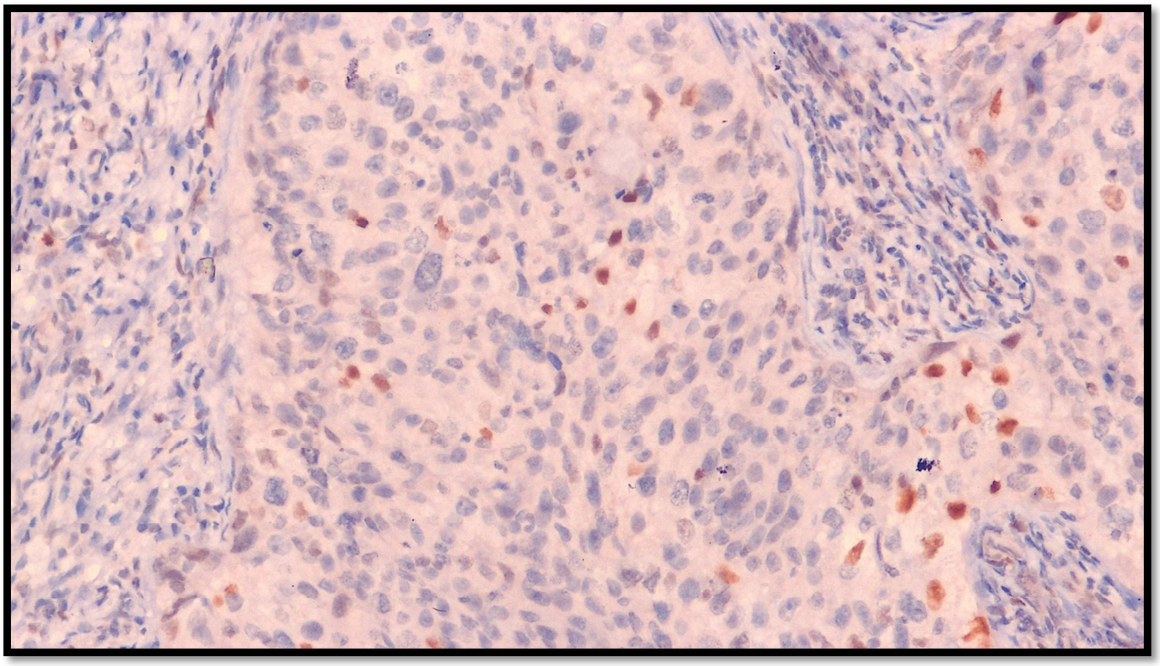


Pictomicrograph 6 – Poorly differentiated SCC (H&E, 400x)



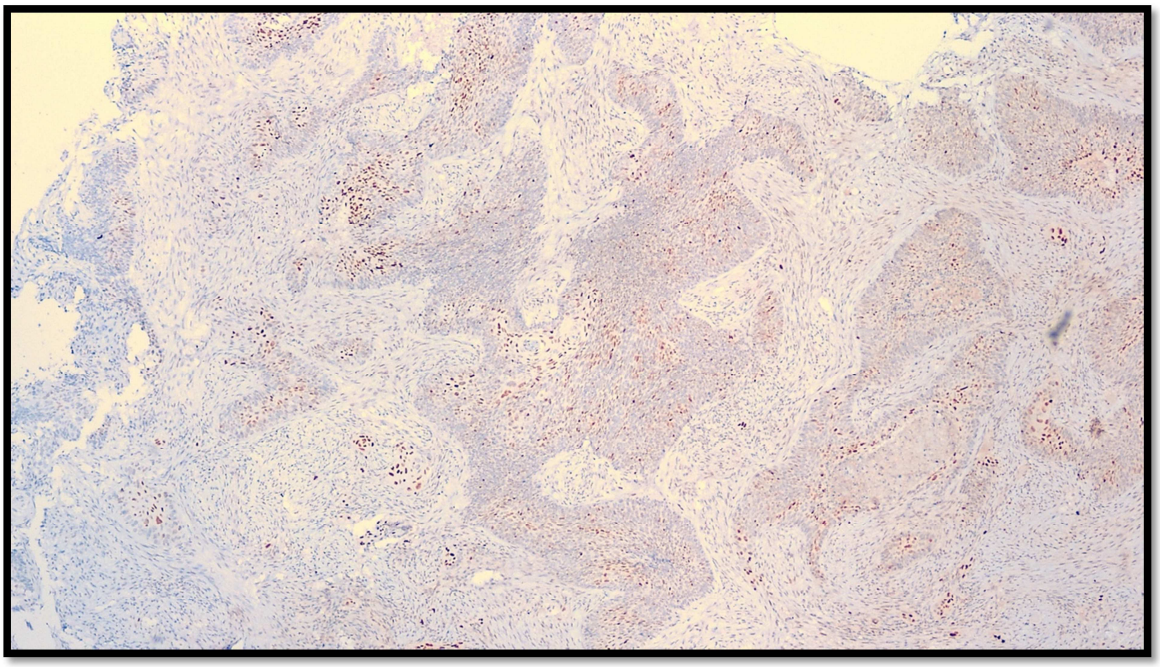
Photomicrograph 7 - Shows moderately differentiated SCC with SOX2 score 3

(IHC, 40x)



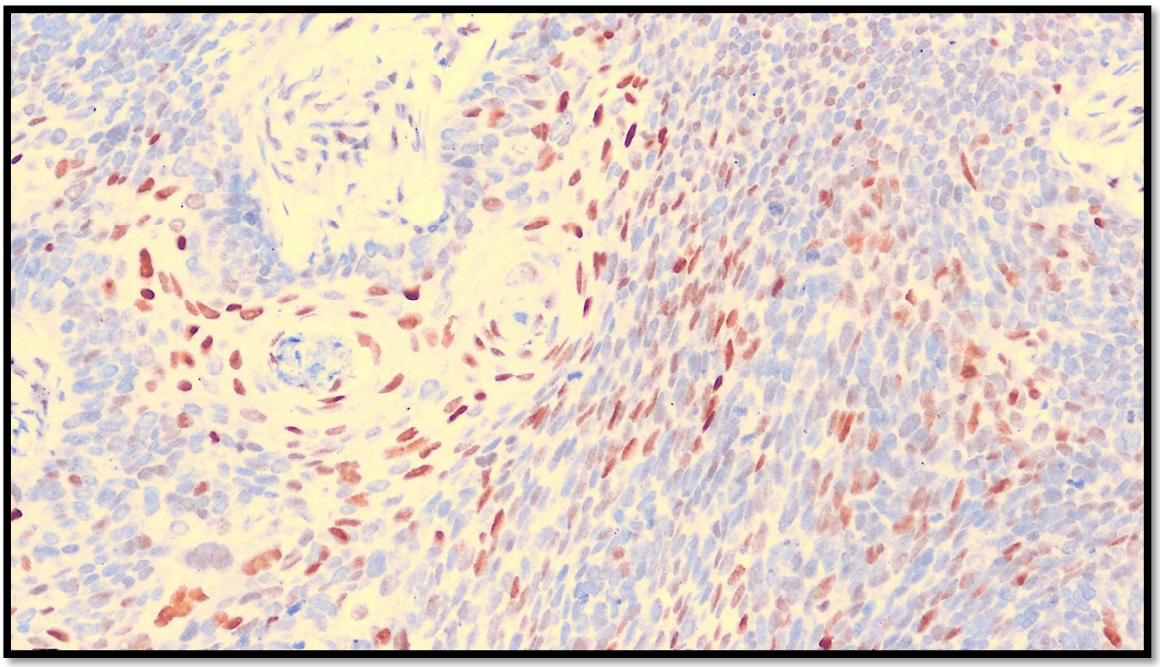
Photomicrograph 8 - Shows moderately differentiated SCC with SOX2 score 3

(IHC, 400x)



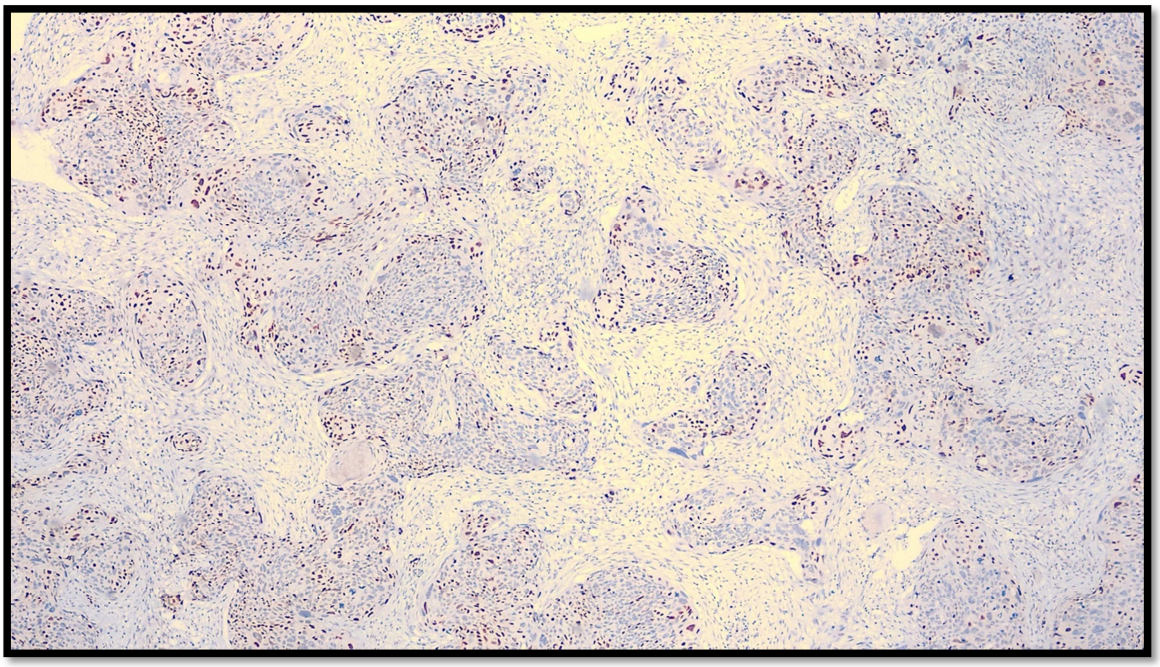
Photomicrograph 9 - Shows moderately differentiated SCC with SOX2 score 6

(IHC, 40x)



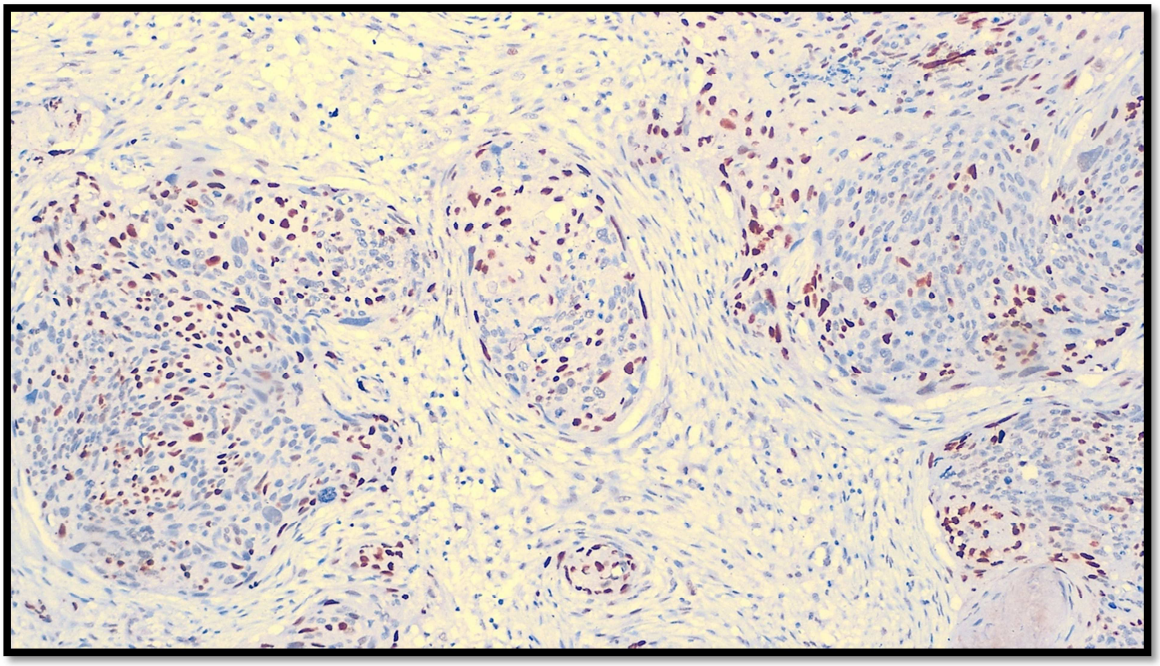
Photomicrograph 10 - Shows moderately differentiated SCC with SOX2 score 6

(IHC, 400x)



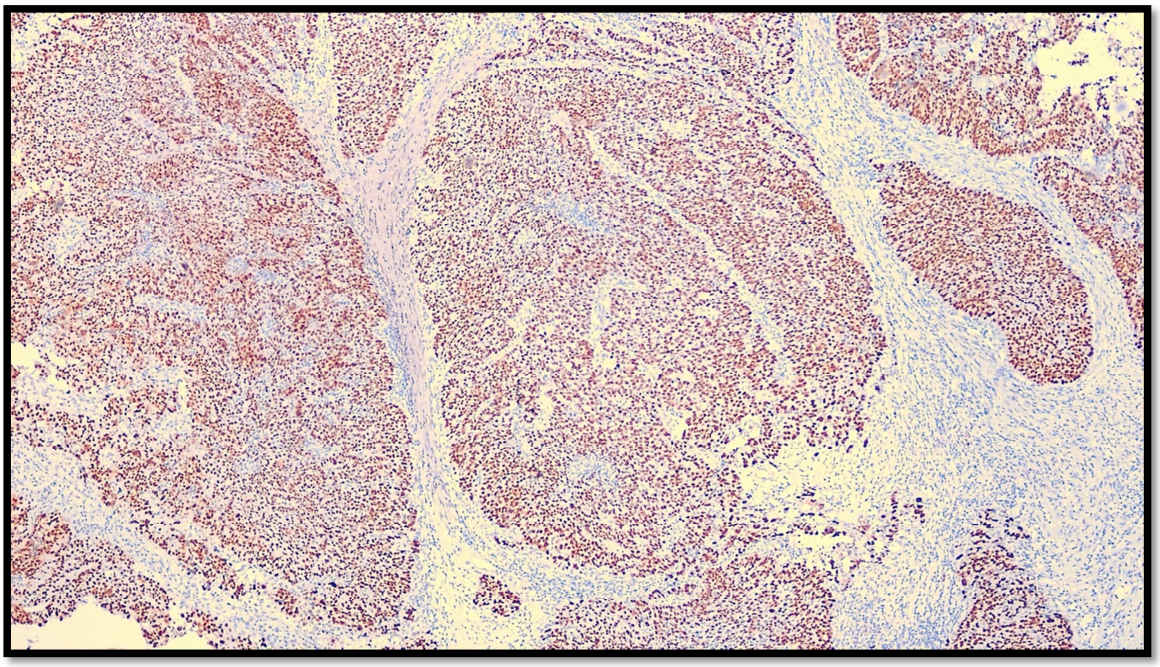
Photomicrograph 11 - Shows moderately differentiated SCC with SOX2 score 9

(IHC, 40x)



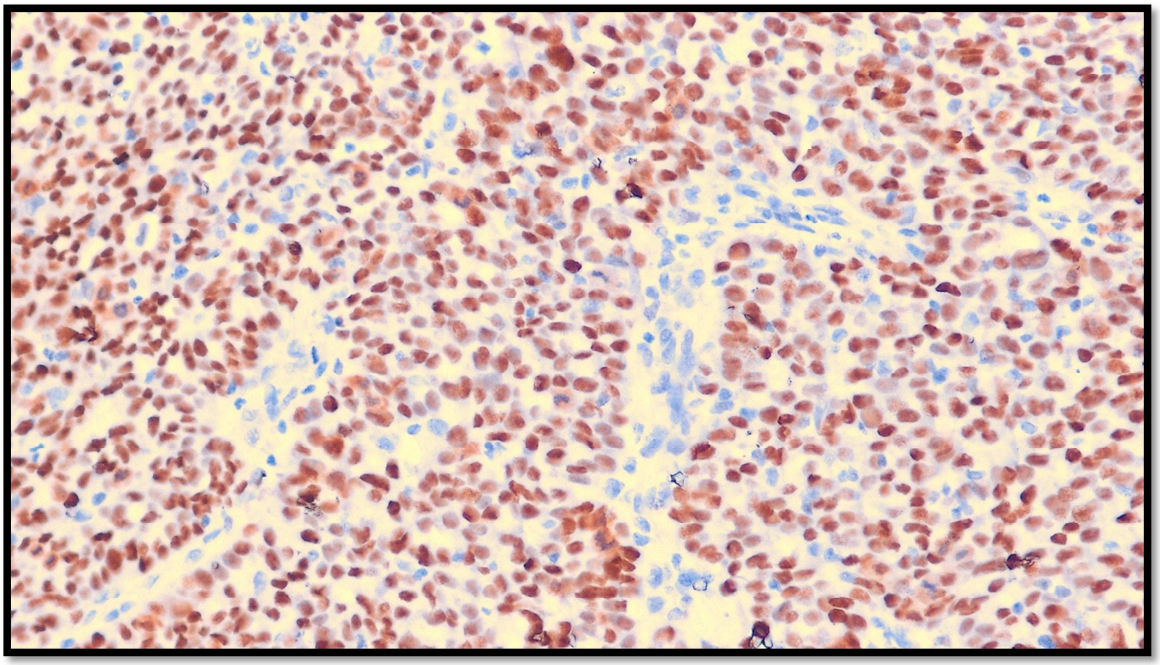
Photomicrograph 12 - Shows moderately differentiated SCC with SOX2 score 9

(IHC, 400x)



Photomicrograph 12 - Shows moderately differentiated SCC with SOX2 score 12

(IHC, 40x)



Photomicrograph 13 - Shows moderately differentiated SCC with SOX2 score 12

(IHC,400x)

DISCUSSION

Cervical carcinoma accounts for more than 3% of the global deaths and squamous cell carcinoma is the most common subtype and one of the leading causes of morbidity and mortality especially in developing countries[72]. In India cervical cancer accounts for 17% of deaths between the age group of 30 to 69[2].

SOX2 protein, a product of the SRY gene is a transcription factor that plays an important role in tumorigenesis in various cancers[44]. Its weak positive expression is seen in 20% of normal cervix but is known to be more prevalent in cervical squamous cell carcinomas[73]. Since SOX2 is known to play an important role in the pathogenesis of stratified squamous carcinomas of the GIT[53]. High expression is also known to be associated with aggressive behavior in stage 1 lung adenocarcinomas[7].

In cervical squamous cell carcinoma, there is lack of consensus on extent of SOX2 expression as shown in table 14. Some studies like Maier et. al have found low SOX2 expression while other studies like Shel et. al have found 100% SOX2 expression in cervical squamous cell carcinoma. There is also lack of consensus if SOX2 plays a role in differentiation of these carcinomas as some studies show correlation of SOX2 with poor differentiation while others show no correlation[8,63]. Dayalan et. al found a positive association of SOX2 and p16 in cervical squamous cell carcinomas further supporting the fact that SOX2 plays an essential role in the pathogenesis of squamous cell carcinoma[64].

Shen et. al illustrated a correlation between SOX2, OCT4 expression and resistance to radiotherapy as they are stem cell markers[63]. Thus, SOX2 has the potential to be used for prognosis in conjunction with OCT4. SOX2 also has the

potential to be a therapeutic target to prevent relapse by cancer stem cells. Dayalan et. al found a positive association of SOX2 and p16 in cervical squamous cell carcinomas further supporting the fact that SOX2 plays an essential role in the pathogenesis of squamous cell carcinoma.

The purpose of this study is to estimate the extent of SOX2 expression in cervical squamous cell carcinomas and to correlate this expression with histological grading. In this study 50 histologically diagnosed cervical squamous cell carcinomas were studied to estimate their SOX2 expression.

The mean age was 53.44 years and age showed no correlation with SOX2 expression. These findings correlated with Z. Yang et. al who also found no correlation between age and extent of SOX2 expression[8].

All the 50 cases of cervical squamous cell carcinomas were graded using Broder's grading which is based on morphology of squamous cells, keratin pearls, mitotic figures and nuclear pleomorphism. This study showed that most of the cases were moderately differentiated squamous cell carcinomas (76%).

IHC scoring was done on the basis of the number of positive cells and the intensity of stain uptake by the nucleus. 48 of the 50 cases showed positivity and the observations from other studies are summarized and compared in table 14.

Table 16 – Studies with SOX2 positivity in cervical squamous cell carcinoma.

Study	Technique	Percentage of SCC with SOX2 positivity
Yang(8)	IHC	74.5% (41/55)
Kim et. al(74)	IHC	77.63% (125/161)
Dayalan(64)	IHC	84% (16/19)
Shen(63)	IHC	100% (132/132)
Stewart et. al(73)	IHC	53% (8/15)
Maier et. al(75)	FISH	27% (13/47)
Zheng(57)	Cell culture and IHC	77.5% (31/40)
Present study	IHC	96% (48/50)

The variation in various studies could be because of different fixation, antigen retrieval methods, scoring systems used having different cutoffs for positive SOX2 expression.

This study supports the idea that SOX2 is expressed by squamous cell carcinoma cells more readily and intensely compared to normal cervix epithelium. Zheng and Jing found correlation between SOX2 and grades of differentiation but Yang et. al and other studies including ours did not find any significant positive or negative association between SOX2 expression and differentiation of squamous cell carcinomas.

CONCLUSION

This study evaluated SOX2 expression in 50 cervical squamous cell carcinomas and the expression was correlated with histological parameters like Broder's grading. The percentage of tumor cells were determined along with intensity of staining and a final IHC score was given. 48 (96%) of the 50 cases showed positivity for SOX2 expression with 4% negative, 42% weak, 28% medium and 26% with strong expression. No correlation was found between Broder's grade for differentiation and SOX2 expression.

SUMMARY

In the present study, 50 histopathologically diagnosed cases of cervical squamous cell carcinomas were studied from the time period January 2021 to December 2021 at Dept of Pathology, JNMC, Belagavi. Most of the cases (90%) in this study were punch biopsies, 3 hysterectomy and 1 endocervical curettage specimens were also included.

Paraffin embedded blocks of all 50 cases were subjected to immunohistochemical staining for SOX2 and its result was correlated with clinicopathological parameters.

The peak incidence was between 55-64 years with the mean and median age of 53 years and 55 years respectively. Most of the cases were moderately differentiated carcinomas according to Broder's grading system.

98% or 48 out of 50 cases of SCC cervix in this study have shown positivity for SOX2 immunostaining. However, cases varied in intensity of staining and percentage positive tumor cells.

A majority of the cases showed strong intensity of SOX2 expression and also was positive in more than 25% of the tumor positive cells in most cases.

The overall IHC expression score was predominantly weak seen in 42% of the cases. There was no significant correlation found between IHC score and age or between Broder's grade and percentage of positive cells or intensity. No correlation was found between Broder's grade and IHC even when each grade group was evaluated individually.

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ANNEXURE-I

WHO CLASSIFICATION OF CERVIX TUMOURS, 2020, 5th Edition

SQUAMOUS EPITHELIAL TUMOURS

1. Mimics of squamous precursor lesions
    a. Squamous metaplasia
    b. Atrophy

2. Squamous cell tumours and precursors
    a. Condyloma acuminatum
    b. Squamous intraepithelial lesions
    c. Squamous cell carcinoma, HPV-associated
    d. Squamous cell carcinoma, HPV-independent
    e. Squamous cell carcinoma NOS

GLANDULAR TUMOURS AND PRECURSORS

1. Benign glandular lesions
    i. Endocervical polyp
    ii. Müllerian papilloma
    iii. Nabothian cyst
    iv. Tunnel clusters
    v. Microglandular hyperplasia
    vi. Lobular endocervical glandular hyperplasia
    vii. Diffuse laminar endocervical hyperplasia
    viii. Mesonephric remnants and hyperplasia
    ix. Arias-Stella reaction

x. Endocervicosis

xi. Tuboendometrioid metaplasia

xii. Ectopic prostate tissue

2. Adenocarcinomas

i. Adenocarcinoma in situ, HPV-associated

ii. Adenocarcinoma, HPV-associated

iii. Adenocarcinoma in situ, HPV-independent

iv. Adenocarcinoma, HPV-independent, gastric type

v. Adenocarcinoma, HPV-independent, clear cell type

vi. Adenocarcinoma, HPV-independent, mesonephric type

vii. Other adenocarcinomas

OTHER EPITHELIAL TUMOURS

1. Carcinosarcoma
2. Adenosquamous and mucoepidermoid carcinomas
3. Adenoid basal carcinoma
4. Carcinoma, unclassifiable

MIXED EPITHELIAL AND MESENCHYMAL TUMOURS

1. Adenomyoma
2. Adenosarcoma

GERM CELL TUMOURS

ANNEXURE-II

REVISED FIGO STAGING OF CERVICAL CARCINOMA (2018)

1. FIGO no longer includes stage 0 (Tis)
2. **I:** confined to cervix uteri (extension to the corpus should be disregarded)
    a. **IA:** invasive carcinoma only diagnosed by microscopy
        i. **IA1:** stromal invasion <3 mm in depth
        ii. **IA2:** stromal invasion ≥3 mm and <5 mm in depth
    b. **IB:** invasive carcinoma with measured deepest invasion ≥5 mm (greater than stage IA), lesion limited to the cervix uteri
        i. **IB1:** invasive carcinoma ≥5 mm depth of stromal invasion and <2 cm in greatest dimension
        ii. **IB2:** invasive carcinoma ≥2 cm and <4 cm in greatest dimension
        iii. **IB3:** invasive carcinoma ≥4 cm in greatest dimension
3. **II:** beyond the uterus, but has not extended onto the lower third of the vagina or to the pelvic wall
    a. **IIA:** involvement limited to the upper 2/3 of vagina without parametrial invasion
        i. **IIA1:** invasive carcinoma <4 cm in greatest dimension.
        ii. **IIA2:** invasive carcinoma ≥4 cm in greatest dimension
    b. **IIB:** with parametrial involvement but not up to the pelvic wall

4. **III:** carcinoma involves the lower third of the vagina and/or extends to the pelvic wall and/or causes hydronephrosis or non-functioning kidney and/or involves pelvic and/or paraaortic lymph nodes
    a. **IIIA:** carcinoma involves the lower third of the vagina, with no extension to the pelvic wall
    b. **IIIB:** extension to the pelvic wall and/or hydronephrosis or non-functioning kidney (unless known to be due to another cause)
    c. **IIIC:** involvement of pelvic and/or para-aortic lymph nodes, irrespective of tumor size and extent
        i. **IIIC1:** pelvic lymph node metastasis only
        ii. **IIIC2:** para-aortic lymph node metastasis
        iii. with r (imaging) and p (pathology) notations to indicate how lymph nodes were identified
5. **IV:** carcinoma has extended beyond the true pelvis or has involved (biopsy-proven) the mucosa of the bladder or rectum (bullous edema, as such, does not permit a case to be allotted to stage IV)
    a. **IVA:** spread to adjacent organs
    b. **IVB:** spread to distant organs

ANNEXURE-III

TNM STAGING 2021 (AJCC VERSION 9)

PRIMARY TUMOR (T)

TNM ***Categories***	Surgical-Pathologic Findings
TX	Primary tumor cannot be assessed
T0	No evidence of primary tumor
Tis	Carcinoma in situ (preinvasive carcinoma)
T1	Cervical carcinoma confined to the cervix (disregard extension to the corpus)
T1a	Invasive carcinoma diagnosed only by microscopy; stromal invasion with a maximum depth of < 5.0 mm, measured from the base of the epithelium; vascular space involvement, venous or lymphatic, does not affect classification
T1a1	Measured stromal invasion < 3.0 mm in depth
T1a2	Measured stromal invasion ≥ 3.0 mm and < 5.0 mm
T1b	Invasive carcinoma with measured deepest invasion ≥ 5 mm (greater than stage IA), lesion limited to the cervix
T1b1	Invasive carcinoma with ≥ 5 mm depth of stromal invasion and < 2 cm in greatest dimension
T1b2	Invasive carcinoma, 2 cm to < 4 cm in greatest dimension
T1b3	Invasive carcinoma, ≥ 4 cm in greatest dimension
T2	Cervical carcinoma invades beyond uterus but not to pelvic wall or to lower third of vagina

T2a	Involvement limited to the upper two-thirds of the vagina, without parametrial invasion
T2a1	Invasive carcinoma < 4 cm in greatest dimension
T2a2	Invasive carcinoma ≥ 4 cm in greatest dimension
T2b	Tumor with parametrial invasion but not up to the pelvic wall
T3	Carcinoma involves the lower third of the vagina and/or extends to the pelvic wall and/or causes hydronephrosis or nonfunctioning kidney and/or involves pelvic and/or para-aortic lymph nodes
T3a	Tumor involves lower third of vagina, with no extension to pelvic wall
T3b	Tumor extends to pelvic wall and/or causes hydronephrosis or nonfunctional kidney
T3c	Involvement of pelvic and/or para-aortic lymph nodes, irrespective of tumor size and extent (with r [imaging] and p [pathology] notations)
T3c1	Pelvic lymph node metastasis only
T3c2	Para-aortic lymph node metastasis
T4	The carcinoma has extended beyond the true pelvis or has involved (biopsy proven) the mucosa of the bladder or rectum. (A bullous edema, as such, does not permit a case to be allotted to stage IV.)
	Spread to adjacent pelvic organs
	Spread to distant organs

REGIONAL LYMPH NODES (N)

NX	Regional lymph nodes cannot be assessed
N0	No regional lymph node metastasis
N0 (i+)	Isolated tumor cells in regional lymph node(s) ≤ 0.2 mm
N1	Regional lymph node metastasis

DISTANT METASTASIS (M)

M0	No distant metastasis
M1	Distant metastasis (including peritoneal spread; involvement of supraclavicular, mediastinal, or distant lymph nodes; and lung, liver, or bone)

AJCC PROGNOSTIC GROUPS

Stage	Tumor	Node	Metastasis
I	T1	Any N	M0
IAI	T1a	Any N	M0
IA2	T1a1	Any N	M0
IB	T1b	Any N	M0
IB1	T1b1	Any N	M0
IB2	T1b2	Any N	M0
II	T2	Any N	M0
IIA	T2a	Any N	M0
IIA1	T2a1	Any N	M0
IIA2	T2a2	Any N	M0
IIB	T2b	Any N	M0
III	T3	Any N	M0
IIIA	T3a	Any N	M0
IIIB	T3b	Any N	M0
IVA	T4	Any N	M0
IVB	Any T	Any N	M1

ANNEXURE IV

INFORMED CONSENT FORM

ESTIMATING PREVALENCE OF SOX-2 EXPRESSION IN CERVICAL SQUAMOUS CARCINOMAS – A HOSPITAL BASED CROSS SECTIONAL STUDY

The principal investigator of the study is Dr. _____ under the guidance of Dr. _____ (guide).

Purpose of the study: The purpose of this study is to estimate correlation of SOX-2 expression in cervical squamous cell carcinomas with histological grading. You are being asked to enrol in this study as you are eligible for participation in this study. If you undergo cervical biopsy or hysterectomy you will be included in this study.

Procedure: During this study, you will be asked questions regarding history and background and you are supposed to answer to the best of your knowledge. If you agree to enrol yourself in this study, you will be interviewed regarding your present, past and family history and your clinical manifestations.

Risks and benefits: There are no risks involved in taking part in this study and benefit is we will be able to know a better method for diagnosis of cervical squamous carcinoma which is essential for providing appropriate treatment.

Alternatives: Taking part in this study is voluntary. You may choose not to take part in this study or if you decide to take part now, you can later change your mind and withdraw from the study. The study doctor may terminate your participation in this study anytime.

Privacy and confidentiality: All information collected about you during the course of this study will be kept confidential to the extent permitted by law. The code numbers will identify you in this research record. Information from this study will be published but your identity will be confidential in any publication. No information about you or information provided by you during research will be disclosed to other without your written permission except:

1. In emergency to protect your rights and welfare.
2. If required by law.

Financial incentives for participation: You will not be paid / offered any gift /incentives for participating in this study.

Authorization to publish results: The results of this study would be forwarded to the KAHER University, Belagavi as a part of requirement towards the completion of MD degree, review and publishing.

Questions: In case you have any questions related to the study in future you can contact:

1. If you have any queries about your rights as a study subject, you may call Dr. Roopa Bellad, Professor, Department of Paediatrics, Chairman of J.N. Medical College Institutional Ethical Committee of Human Subjects Research, Ph No- 9448113403, at J.N. Medical College, Belagavi

CONSENT STATEMENT

I voluntarily agree to take part in this study by signing below. I may withdraw at any time. I am not giving up any legal rights by signing this form. My signature below indicates that I have read, or it has been read to me, this entire consent form and have had all my questions answered.

Name of the participant:

(signature/thumbprint)

Name of the witness :

(signature/thumbprint)

Name of the investigator:

(signature)

Date :

ANNEXURE V

PROFORMA

PATIENT HISTORY

Name: Age:

Date :

IP no.:

BRIEF CLINICAL HISTORY :

PAST HISTORY :

History of sexually transmitted diseases

GYNAECOLOGICAL HISTORY:

Menstrual history :

Age of menarche

Length of cycle.

Age of menopause

History of contraceptive use

OBSTETRIC HISTORY:

FAMILY HISTORY:

PERSONAL HISTORY:

Age of marriage

History of HPV vaccination

EXAMINATION FINDINGS

Per vaginal examination:

Colposcopy findings :

Pap smear findings:

CLINICAL DIAGNOSIS :

HISTOPATHOLOGICAL DIAGNOSIS :

1. **Grading on Hematoxylin and Eosin staining :**

2. **IHC staining:**

MARKER	**Fraction of positive cells**	**Grading of immunoreactivity.**
SOX2		

ANNEXURE-VI

HEMATOXYLIN AND EOSIN STAINING PROTOCOL

1. Deparaffinize in Xylene I and II and III changes. (III change use warmed xylene) (5 minutes in each)
2. Rehydrate using
    a. Absolute ethanol 100% (5 minutes)
    b. Absolute Ethanol 100% (5 minutes)
3. Rinse in distilled water (5 minutes)
4. Rinse in running tap water (5 minutes)
5. Stain in Harris's haematoxylin by progressive method (2 minutes) Fresh and filtered
6. Rinse in running tap water (20 minutes)
7. Decolorize in 1% acid alcohol (1 second)
8. Rinse well in tap water (5 minutes)
9. Immerse in hot water bath, 55˚C for blueing (3 seconds)
10. Rinse in tap water (5 minutes)
11. Counterstain in Eosin (15 seconds)
12. Dehydrate with absolute alcohol 100% (2-4 dips)
13. Clear in xylene I and II (5 minutes)
14. Mount with DPX.

Stock solution – Eosin:

Stock – 1% aqueous Eosin – Y

Stock – 1% aqueous Phloxin B

Working Solution – Eosin:

100ml stock Eosin

10 ml stock Phloxin B

780 ml 95% Ethanol

4 ml glacial acetic acid

Working Solution – Hematoxylin

Harris Hematoxylin, 1 litre

Working solution – 0.25% Acid alcohol

95% Ethanol, 2578 ml

dH2O, 950 ml

HCl, 9ml

Result: Nuclei – blue, cytoplasm – pink, RBCs – red.

Reference: Bancroft D, Laylon C. The haematoxylin and eosin, In: Kim SS Ed, Bancroft's Theory and practice of histopathological techniques. &th Ed., China, Churchill Livingstone; 2013: p173-187.

PROCEDURE FOR IHC STAINING FOR SOX2 ANTIBODY

1. Cut the sections at approximately 3-4 µm thickness in poly L Lysine coated slides.
2. Float on to the positive charged slides.
3. Slides were air dried for 2 hours at 58 ºC.
4. Two changes of xylene of 10 minutes each for deparaffinization.
5. Hydration:
    a) Absolute alcohol - 2 dips
    b) 80% alcohol - 2 dips
    c) 70% alcohol - 2 dips
    d) Distilled water - 2 changes 5 minutes each.
6. Antigen retrieval by heat, using microwave and TRIS EDTA Buffer.
7. Cooling of sections to room temperature.
8. Rinse in distilled water for 3 minutes.
9. Wash in Tris buffered saline **(**TBS) buffer two times for 3 minutes each.
10. Treatment with peroxide block for 10 minutes to block endogenous peroxidase.
11. Wash in TBS buffer two times for 3 minutes each.
12. Treatment with primary antibody (SOX2) for 60 minutes
13. Wash in TBS buffer two times for 3 minutes each
14. Treatment with Target binder for 10 minutes
15. Wash in TBS buffer two times for 3 minutes each
16. Treatment with HRP Polymer for 10 minutes
17. Wash in TBS buffer two times for 3 minutes each

18. Treatment with DAB (secondary antibody) for 3-5 minutes to give brown colour to antigens
19. Wash in distilled water for 3 minutes
20. Counter stain with Harris haematoxylin for 30 seconds to 1 minute
21. Wash in tap water for 3 minutes to remove excess stain
22. Two changes of absolute alcohol for 2 minutes each for dehydration
23. Clearing with xylene for two minutes. Dry the slides and mount with DPX

PREPARATION OF REAGENTS

1. Antigen retrieval Buffer

TRIS EDTA Buffer- pH: 8.5 to 9.0

Preparation:

TRIS Base- 1.21 gram

EDTA (atomic number: 372)- 0.37 gram

Dissolve in 1000ml of water

2. Wash buffer

TRIS BUFFERED SALINE (TBS)- pH: 7.2 to 7.6

Preparation:

TRIS Base- 8.6 gram

NaCl- 9.6 gram

Dissolve in 1000ml of water.

Adjust pH by using concentrated HCl

ANNEXURE VII- MASTER CHART FOR CERVICAL SQUAMOUS CELL CARCINOMA-

SL No.	IP NO.	Age (yr)	Sex	Sample	Specimen type	Type of carcinoma	Broder's grade	% positive	Intensity	IHC Score (%pos X inten.)
GROUP 1										
1	13663?	70	F	HP/1847/21	Punch biopsy	Keratinizing well differentiated Squamous cell carcinoma	1	2	2	4
2	1000138	60	F	HP/0519/20	Punch biopsy	Large cell well Keratinizing Squamous cell carcinoma	1	2	2	4
3	987555	52	F	HP/5762/19	Punch biopsy	Well differentiated Squamous cell carcinoma	1	1	3	3
4	21631?	55	F	HP/2928/18	Punch biopsy	Well differentiated Squamous cell carcinoma	1	2	3	6

5	884621	56	F	HP/3671/18	Punch biopsy	Well differentiated keratinizing Squamous cell carcinoma	1	2	3	6
6	17112?	37	F	HP/1690/20	Punch biopsy	Well differentiated Squamous cell carcinoma	1	3	3	9
GROUP 2										
7	1875325	50	F	HP/2174/21	Punch biopsy	CIN3 with moderately invasive squamous cell carcinoma	2	2	3	6
8	1846521	60	F	HP/1900/21	Hysterectomy	Moderately differentiated squamous cell carcinoma	2	1	1	1
9	1853472	49	F	HP/1872/21	Punch biopsy	Moderately differentiated squamous cell	2	3	3	9

						carcinoma				
10	1111223	53	F	HP/1670/21	Punch biopsy	Moderately differentiated squamous cell carcinoma	2	1	3	3
11	1104900	60	F	HP/1432/21	Punch biopsy	Moderately differentiated squamous cell carcinoma	2	4	3	12
12	1106524	55	F	HP/1326/21	Punch biopsy	Moderately differentiated squamous cell carcinoma	2	1	3	3
13	1831325	38	F	HP/1276/21	Punch biopsy	Moderately differentiated squamous cell carcinoma	2	3	3	9
14	182431	46	F	HP/1205/21	Punch biopsy	Moderately differentiated squamous cell carcinoma	2	2	2	4

15	1045545	56	F	HP/893/21	Punch biopsy	Moderately differentiated squamous cell carcinoma	2	1	3	3
16	1033701	63	F	HP/029/21	Endocervical curettage	Moderately differentiated squamous cell carcinoma	2	2	2	4
17	1046290	60	F	HP/945/21	Punch biopsy	Moderately differentiated Squamous cell carcinoma	2	2	3	6
18	8859?	55	F	HP/1097/21	Punch biopsy	Moderately differentiated Squamous cell carcinoma	2	2	3	6
19	1040988	57	F	HP/519/21	Punch biopsy	Keratinizing moderately differentiated Squamous cell carcinoma	2	2	3	6

20	1064479	47	F	HP/1881/21	Punch biopsy	Moderately differentiated Squamous cell carcinoma	2	1	3	3
21	1065559	55	F	HP/1932/21	Punch biopsy	Moderately differentiated Squamous cell carcinoma	2	3	3	9
22	1084346	55	F	HP/3076/21	Punch biopsy	Moderately differentiated squamous cell carcinoma	2	0	0	0
23	1013253	68.	F	HP/1177/20	Punch biopsy	Moderately differentiated large cell non-keratinizing Squamous cell carcinoma	2	2	3	6
24	1012855	52	F	HP/1179/20	Punch biopsy	Moderately differentiated large cell non-keratinizing Squamous cell carcinoma	2	3	2	6

25	1017083	65	F	HP/1388/20	Punch biopsy	Moderately differentiated Squamous cell carcinoma	2	2	3	6
26	1016203	48	F	HP/1403/20	Punch biopsy	Moderately differentiated Squamous cell carcinoma	2	1	3	3
27	931936	45	F	HP/1431/19	Punch biopsy	Invasive moderately differentiated squamous cell carcinoma	2	3	3	9
28	1031530	40	F	HP/2071/20	Punch biopsy	Moderately differentiated Squamous cell carcinoma	2	3	3	9
29	932381	65	F	HP/1526/19	Punch biopsy	Moderately differentiated non-keratinizing Squamous cell carcinoma	2	3	3	9

30	942578	42	F	HP/2619/19	Punch biopsy	Moderately differentiated Squamous cell carcinoma	2	3	2	6
31	947436	42	F	HP/3099/19	Hysterectomy	Moderately differentiated non-keratinizing Squamous cell carcinoma	2	1	2	2
32	959009	63	F	HP/4077/19	Punch biopsy	Moderately differentiated keratinizing Squamous cell carcinoma	2	1	3	3
33	959072	60	F	HP/4078/19	Punch biopsy	Moderately differentiated keratinizing Squamous cell carcinoma	2	0	0	0
34	30923?	55	F	HP/4508/19	Punch biopsy	Moderately differentiated Squamous cell carcinoma	2	1	3	3

35	971714	40	F	HP/4768/19	Punch biopsy	Moderately differentiated keratinizing Squamous cell carcinoma	2	3	2	6
36	978561	60	F	HP/5383/19	Punch biopsy	Moderately differentiated Squamous cell carcinoma	2	1	3	3
37	978106	45	F	HP/5382/19	Punch biopsy	Large cell non-keratinizing moderately differentiated Squamous cell carcinoma	2	3	3	9
38	851332	55	F	HP/0247/18	Punch biopsy	Moderately differentiated Squamous cell carcinoma	2	2	3	6
39	855969	45	F	HP/0674/18	Punch biopsy	Moderately differentiated Squamous cell carcinoma	2	2	2	4

40	14392?	56.	F	HP/1822/18	Punch biopsy	Moderately differentiated Squamous cell carcinoma	2	2	3	6
41	867785	48	F	HP/1854/18	Punch biopsy	Moderately differentiated Squamous cell carcinoma	2	4	3	12
42	889360	50	F	HP/4102/18	Punch biopsy	Moderate differentiated keratinizing Squamous cell carcinoma	2	4	3	12
43	891171	55	F	HP/4246/18	Punch biopsy	Moderate differentiated squamous cell carcinoma	2	1	2	2
44	3026621	40.	F	HP/1824/21	Hysterectomy	Moderately differentiated squamous cell carcinoma	2	2	2	4

GROUP 3										
45	1855312	50	F	HP/1941/21	Punch biopsy	Poorly differentiated carcinoma	3	3	2	6
46	5098243	63	F	HP/1581/21	Punch biopsy	Poorly differentiated squamous cell carcinoma	3	4	3	12
47	1051970	70	F	HP/1379/21	Punch biopsy	Poorly differentiated squamous cell carcinoma	3	2	2	4
48	1072780	72	F	HP/2405/21	Punch biopsy	Poorly differentiated neoplasm	3	4	3	12
49	1079298	49	F	HP/2901/21	Hysterectomy	Poorly differentiated Squamous cell carcinoma	3	2	2	4
50	858231	40	F	HP/0847/18	Punch biopsy	Poorly differentiated squamous cell carcinoma	3	1	2	2