A STANDARDISED FRACTION 1 (SF1) FROM *Clinacanthus nutans* ATTENUATES THE STEMNESS OF CERVICAL CANCER STEM-LIKE CELLS VIA INHIBITION OF NOTCH1 SIGNALLING

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LIST OF SYMBOLS

%	percentage
<	less than
>	greater than
=	equal
/	per
-	minus
+	plus
±	plus minus
^{o}C	degree Celsius
µg	microgram
µg/mL	microgram per millilitre
µL	microliter
µm	micrometre
µM	micromolar
cm	centimetre
Co2	carbon dioxide
g	gram
g/kg	gram per kilogram
g/kg/day	gram per kilogram per day
h	hour
IC_{50}	inhibitory concentration 50%
kDa	kilodaltons
LD_{50}	lethal dose 50%
m	meter

mg	milligram
mg/kg	milligram per kilogram
mg/kg/day	milligram per kilogram per day
mL	millilitre
mL/cm^2	millilitre to square centimetre
n	number
ng/mL	nanogram per millilitre
nM	nanomolar
nmol/L	nanomole per litre
R^2	R-squared
rpm	revolutions per minute
x	multiplication
x g	relative centrifugal force

LIST OF ABBREVIATIONS

2D	two-dimensional
3D	three-dimensional
A549	human lung carcinoma epithelial cell line
ABC	ATP-binding cassette
ABTS	2,2-azinobis (3-ethylbenzothiazoline-6-sulfonic acid)
AC	adenocarcinoma
ADAM	a disintegrin and metalloprotease
Akt	protein kinase B
ALDH	aldehyde dehydrogenase
ANOVA	one-way analysis of variance
ATCC	American Type Culture Collection
ATRA	All-Trans Retinoids Acid
β-actin	beta-actin
B27	neuronal culture supplement
Bax	apoptosis regulator protein
Bcl-2	apoptosis deregulator protein
BD	Becton Dickinson
bFGF	basic fibroblast growth factor
BME	2-mercaptoethanol
BSA	bovine serum albumin
BSC	biosafety cabinet
C. nutans	*Clinacanthus nutans*
C-33A	human cervical cancer cell line (HPV-negative)
C-4 I	human cervical cancer cell line

CaSki	human cervical cancer cell line (HPV 16- positive)
CCSC	cervical cancer stem cell
CD44	cluster of differentiation 44
CD49f	integrin α6
CD133	prominin-1
CECU	*Citrus unshiu Markovich* peel
cDNA	complementary DNA
CIN	cervical intraepithelial neoplasia
CK17	cytokeratin-17
CNE1	human nasopharyngeal cancer cell line
COVID-19	coronavirus disease of 2019
CP	cisplatin
CRT	concurrent chemoradiation therapy
CS	cervospheres
CSC	cancer stem cell
Ct	cycle threshold
DAB	3,3′-Diaminobenzidine
DLL	delta-like ligand
DMEM	Dulbecco’s Modified Eagle’s Medium
DMEM: F12	Dulbecco's Modified Eagle's Medium/Nutrient Mixture F12 Ham
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DPPH	2,2-diphenyl-1-picrylhydrazyl
EDTA	ethylenediamine tetra acetic acid
EGF	epidermal growth factor
EMT	epithelial-mesenchymal transition

ESC	embryonic stem cell
F	fraction
FACS	fluorescence-activated cell sorting
FBS	fetal bovine Serum
FIGO	International Federation of Gynaecology and Obstetrics
FITC	fluorescein isothiocyanate
FTIR	Fourier transform infrared spectroscopy
G1	gap 1
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GLI1	glioma-associated oncogene homolog 1
GLOBOCAN	Global Cancer Observatory
HeLa	cervical adenocarcinoma cell line (HPV 18-positive)
HepG1/2	human liver cancer cell line
HCI	hydrochloric acid
HEY-1	Hairy/enhancer-of-split related with YRPW motif protein 1
HES-1	Hairy Enhancer of Split 1
HFHC	high-fat and high-cholesterol
Hh	Hedgehog
HIER	heat-induced epitope retrieval
HIV	human immunodeficiency virus
HPLC	high-performance liquid chromatography
HPTLC	high-performance thin-layer chromatography
HPV 16	human papillomavirus type 16
HPV 18	human papillomavirus type 18
HR-HPV	high risk human papillomavirus
HRP	horseradish peroxidase

HSIL	high-grade cervical intraepithelial lesions
HT-29	human adenocarcinoma cells
ICIs	immune checkpoint inhibitors
IFN- γ^{+}	interferon-gamma
IHC	immunohistochemistry
IIUM	International Islamic University Malaysia
IL	interleukin
IMR32	human neuroblastoma cell line
IVC	individual ventilated cage
JAK-STAT	Janus kinase/signal transducers and activators of transcription
K562	human erythroleukemia cell line
L929	mouse fibroblast cell line
LCMS	liquid chromatography-mass spectrometry
LMP	*Licania michauxii* Prance
LPS	lipopolysaccharide
LSCs	leukemic stem cells
LSIL	low-grade cervical intraepithelial lesions
M-IgG Fc BP	mouse immunoglobulin G fragment crystallizable binding protein
M-IgGκ BP	mouse immunoglobulin G kappa light chain binding protein
MAPK	mitogen-activated protein kinase
MCF-7	human breast cancer cell line
MCF10A	non-tumorigenic human breast epithelial cell line
MDA	malondialdehyde
MDA-MB231	human breast cancer cell line
Mdm-2	mouse double minute 2
ME180	human cervical cancer cell line

ML	monolayer
mRNA	messenger RNA
MS	mass spectrometry
MSI1	Musashi-1
mTOR	mammalian target of rapamycin
MW	molecular weight
MyC	proto-oncogene
Nanog	Nanog homeobox
NaOH	sodium hydroxide
NCL-H23	lung cancer cell
NF-κB	nuclear factor kappa B
NICD	Notch intracellular domain
NIH 3T3	mouse embryonic fibroblast cell line
NMR	nuclear magnetic resonance
NO	nitric oxide
NOAEL	no-observed-adverse-effect level
NOD/SCID	nonobese diabetic/severe combined immunodeficiency mice
Notch	neurogenic locus notch homolog protein
NSCLC	non-small cell lung cancer
NTC	no template control
OCP	oral contraceptive pill
Oct4	Octamer-binding protein 4
OD	optical density
OPLC	optimum performance laminar chromatography
OS	orifice
p-AKT	phosphorylated AKT

PI3K	phosphatidylinositol 3-kinase
PBS	phosphate-buffered serum
PCR	polymerase chain reaction
PE	phycoerythrin
pH	power of hydrogen
PKC	protein kinase C
POU5F1	POU class 5 homeobox 1
pRb	retinoblastoma-associated protein
PVDF	polyvinylidene difluoride
qPCR	quantitative polymerase chain reaction
Raji	human Burkitt's lymphoma cell line
Rf	retention factor
RIPA	Radio-Immunoprecipitation Assay
RNA	ribonucleic acid
ROS	reactive oxygen species
RT-qPCR	real time quantitative polymerase chain reaction
S	synthesis
SALL4	Spalt-Like Transcription Factor 4
SASMEC	Sultan Ahmad Shah Medical Centre
SBE	*Scutellariabarbata D. Don* extraction
SCC	squamous cell carcinoma
SCID	severe combined immunodeficiency
SCJ	squamocolumnar junction
SD	standard deviation
SDS-PAGE	odium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
SF1	semi-purified fraction

SFE	sphere-forming efficiency
SFM	serum-free medium
SiHa	human cervical squamous carcinoma cell line (HPV 16-positive)
SMO	smoothed
SNU-1	gastric cancer cell
Sox2	sex determining region Y-box 2
SPF	specific pathogen-free
TBST	Tris-buffered saline with 0.1% Tween 20
TLC	thin-layer chromatography
TLR-4	toll-like receptor 4
TNF	tumour necrosis factor
TP53	tumour protein P53
USA	United States of America
Vero	monkey kidney cell line
WHO	World Health Organization
WNT	Wingless-related integration site

LIST OF APPENDICES

FRAKSI PIAWAIAN 1 (SF1) DARIPADA C*linacanthus nutans* MELEMAHKAN SIFAT STEM SEL MENYERUPAI SEL STEM KANSER SERVIKS MELALUI PERENCATAN ISYARAT NOTCH1

ABSTRAK

Sel stem kanser (CSC) adalah subset kecil sel kanser dengan ciri-ciri pembaharuan diri tidak terkawal, pengisyaratan stem yang aberan, dan potensi tumorigenik yang tinggi. Banyak bukti menunjukkan bahawa CSC berperanan penting dalam pertumbuhan, perebakan, relaps, dan kerintangan terhadap rawatan kanser konvensional seperti radioterapi and kemoterapi. Oleh itu, tumpuan kajian ke atas CSC akan membuka jalan kepada terapi baharu dalam pencegahan dan rawatan kanser. *Clinacanthus nutans* (*C. nutans*), atau lebih dikenali sebagai Daun Belalai Gajah (Sabah Snake Grass), merupakan tumbuhan herba yang tinggi nilai perubatannya di Asia Timur dengan potensi sebagai antitumor bagi pelbagai jenis sel kanser. Walau bagaimanapun, tiada kajian melaporkan kesannya terhadap CSC. Oleh itu, kajian ini bertujuan untuk menyiasat potensi kesan antitumor SF1, iaitu fraksi separa-penulenan daripada *C. nutans*, terhadap CSC yang diperoleh daripada kanser serviks (CCSC) dan mekanisme yang terlibat. Penfraksian berpandukan bioasai digunakan untuk pengasingan SF1 daripada ekstrak daun *C. nutans*. Sel SiHa digunakan dalam pengayaan CCSC melalui pengkulturan sel dalam medium terkondisi dengan CSC (sel sfera kanser serviks). Ciri-ciri stem sel sfera kanser serviks ditentukan melalui asai pembentukan sfera dan analisis silometri aliran untuk penanda stem, iaitu CD49f, CK17, Sox2, Nanog, dan Oct4. Sel sfera kanser serviks kemudian dirawat dengan SF1, dan cisplatin sebagai kawalan positif. Kesan antitumor SF1 terhadap sel sfera kanser serviks dinilai berdasarkan kelangsungan hidup sel, kecekapan membentuk sfera, dan

kapasiti tumorigenik menggunakan kit kelangsungan hidup OZBlue, asai pembentukan sfera, dan asai tumorigenisiti *in vivo*. Mekanisme bagaimana SF1 menghapuskan CCSC ditentukan dengan menilai ekspresi penanda stem terpilih melalui silometri aliran. Selain itu, aktiviti isyarat Notch1, sebagai pengawal utama pembaharuan diri CSC dan tumorigenisiti, diperiksa menggunakan asai Western blot, transcriptas terbalik kuantitatif PCR (RT-qPCR), dan pewarnaan immunohistokimia. Kajian mendapati bahawa sel sfera kanser serviks daripada sel SiHa menunjukkan kecekapan tinggi dalam pembentukan sfera, dan peningkatan ekspresi penanda stem. Selain itu, kajian lanjut mendapati bahawa sel-sel ini mempunyai tumorigenisiti yang lebih tinggi dalam tikus nude dan peningkatan tahap ekspresi protein dan gen Notch1 berbanding sel induknya, SiHa. SF1 berkesan merencatkan kelangsungan hidup sel sfera kanser serviks dan fenotipe stem *in vitro* dan *in vivo*. SF1 didapati mendorong sitotoksisiti yang bergantung kepada kepekatan dalam sel, dengan kepekatan perencatan separuh maksimum (IC_{50}) sebanyak 17.07 μg/ml. Selain itu, rawatan sel sfera kanser serviks dengan SF1 pada dos IC_{50} selama 72 jam merendahkan kecekapan pembentukan sfera dan merencat pertumbuhan tumor dalam tikus xenotransplant. Selain itu, SF1 menurunkan ekspresi penanda stem dalam sel sfera kanser serviks, terutamanya CK17 dan Sox2. Namun, ekspresi Nanog tidak terjejas. Selain itu, perencatan sifat stem sel sfera kanser serviks disertai dengan penurunan ketara dalam tahap ekspresi protein Notch1. Sebaliknya, peningkatan ekspresi gen Notch1 dicatatkan. Ini menunjukkan bahawa SF1 menggalakkan degradasi proteasomal protein Notch1 atau mengganggu proses translasinya. Kesimpulannya, SF1 mempunyai aktiviti antitumor terhadap CSC daripada SiHa, dengan merendahkan sifat stem dan merencatkan isyarat Notch1. Oleh itu, SF1 berpotensi berfungsi sebagai agen terapeutik dalam meningkatkan pengurusan kanser serviks.

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ABSTRACT

Cancer stem cells (CSCs) represent a tiny subset of cancer cells characterised by deregulated self-renewal, aberrant stemness signalling pathways, and a high tumorigenic potential. Mounting evidence indicates that CSCs play a critical role in cancer growth, metastasis, relapse, and resistance to radiotherapy and chemotherapy. Hence, targeting CSCs will provide novel treatments for cancer. *Clinacanthus nutans* (*C. nutans*), or locally known as Sabah snake grass ('daun belalai gajah'), is a well reputed medicinal herb in East Asia that has shown promising anticancer activities in a range of cancer cell types. However, no study has reported its effect on CSCs. Therefore, this study was conducted to determine the antitumour effect of SF1, a semi-purified fraction from *C. nutans*, on CSCs derived from cervical cancer (CCSCs) and the underlying mechanisms. Bioassay guided fractionation was employed for the isolation of SF1 from *C. nutans* leaf extract. The SiHa cell line was used for the enrichment of CCSCs by culturing the cells in CSC-conditioned medium (cervospheres). Stemness characterisation of the cervospheres was performed using a sphere formation assay and flow cytometric analysis of the stem-related markers, including CD49f, CK17, Sox2, Nanog, and Oct4. The cervospheres were then subjected to SF1 treatment, and cisplatin was used as a positive control. The antitumour effects of SF1 on cervospheres were evaluated by assessing cell viability, sphere-forming efficiency, and tumorigenic capacity using the OZ blue cell viability kit, sphere formation assay, and *in vivo* tumorigenicity assay, respectively. The

mechanisms by which SF1 eliminated CCSCs were determined by evaluating the selected stemness marker expressions via flow cytometry. Additionally, Notch1 signalling activity, a key regulator for CSC self-renewal and tumorigenicity was examined using the Western blot, quantitative reverse transcriptase PCR (RT-qPCR), and immunohistochemistry staining assays. The results of the study showed that SiHa derived cervospheres exhibited high sphere-forming efficiency, and increased expressions of the stemness markers. In addition, further investigation revealed that these cells possessed higher tumorigenicity in nude mice and increased levels of Notch1 protein and gene expression compared to the parental SiHa monolayer cells. SF1 effectively inhibited the cervosphere viability and stemness phenotypes *in vitro* and *in vivo*. SF1 was found to induce a concentration-dependent cytotoxicity in the cells, with a half-maximal inhibitory concentration (IC_{50}) of 17.07 µg/ml. Additionally, treatment of cervospheres with SF1 at the IC_{50} dose for 72 hours resulted in a significant reduction in the sphere-forming efficiency and a suppression of tumour growth in xenotransplant mice. Besides, SF1 decreased the expression of stemness markers in the cervospheres, primarily CK17 and Sox2 expressions. Nevertheless, Nanog expression remained unaffected. Apart from that, the inhibition of cervosphere stemness properties was accompanied by a marked decrease in the level of Notch1 protein expression. In contrast, increased expression of the *Notch1* gene was noted. This implies that SF1 may act by promoting proteasomal degradation of Notch1 protein or disrupting its translation process. In conclusion, SF1 possesses antitumor activity against SiHa derived CSCs, which was achieved through attenuation of their stemness properties and inhibition of the Notch1 signalling. Hence, SF1 may serve as a prospective therapeutic agent for improving cervical cancer management.

CHAPTER 1

INTRODUCTION

1.1 Background of study

For decades, cancer has become a significant burden on society across the world, affecting both developed and developing countries. The cumulative incidence and prevalence of cancer continue to rise owing to population growth, population ageing, and poor lifestyle factors including smoking, obesity, and sedentary lifestyles (Sung *et al.*, 2021). Cervical cancer has been recognised as one of the commonnest malignancies of the female reproductive organ globally (World Health Organization (WHO), 2023). In Malaysia, cervical cancer is ranked as the third most prevalent cancer among females. According to recent reports, 1740 women had been diagnosed with cervical cancer in 2020, with 991 succumbing to the disease. Furthermore, Malaysia is currently home to 12.8 million women aged 15 and above who are classified as having an elevated risk of developing cervical cancer (Bruni *et al*., 2023). Despite the advances in treatment modalities for cervical cancer, global cancer statistics have indicated that the morbidity and mortality of the disease remain high (Singh *et al*., 2023).

There is growing evidence that cancer stem cells (CSCs) might be responsible for the lack of success of current cancer therapeutics. CSCs refer to a small population of tumour cells that are highly tumorigenic and contribute significantly to the development, progression, and maintenance of tumours growth. These cells are endowed with stem and progenitor cell properties, including indefinite capacity to self-renew and differentiate to give rise to the heterogeneous phenotype of the tumour cells. Furthermore, CSCs exhibit high metastatic potential and specific phenotypes that

allow them to evade conventional cancer therapies and cell death, including multidrug resistance, epigenetic reprogramming, and tumour microenvironment protection. These aforementioned CSC characteristics thus contribute to treatment failure, cancer recurrence, and distant metastases (Batlle & Clevers, 2017; Chu *et al*., 2020). Besides, conventional cancer treatments such as chemotherapy and radiotherapy are insufficient for eliminating CSC populations since the approaches are based on the assumption that all somatic cells within the tumours exhibit similar malignant potential (Bighetti-Trevisan *et al.,* 2019). These treatments only kill the rapidly dividing cells of the tumour bulk. Although the therapies reduce the size of the tumour, their effects are usually transient, and cancer recurrence remains an ongoing concern for patients (Meerson *et al.,* 2021). Therefore, it is imperative to develop effective therapeutic approaches that specifically target the CSC population in order to enhance the management of cancer.

Recent studies have proposed that the eradication of CSCs could potentially be achieved through the strategic targeting of CSC biomarkers and the stemness-associated pathways. Different biomarkers have been used to identify CSCs in different types of cancer. Furthermore, the discovery of cancer-type-specific biomarkers have offered potential applications as predictive biomarkers in the diagnosis, treatment, and prognosis of cancer (Das *et al.,* 2023). In cervical cancer, CD49f, CK17, Sox2, Nanog, and Oct4 are common biomarkers used to characterise and isolate the CSC populations. Overexpression of these phenotypic markers (CD49f and CK17) and pluripotent markers (Sox2, Oct4, and Nanog) has been linked to increased tumorigenicity, invasiveness, metastases, treatment resistance, and poor prognosis in cervical cancer patients (Bigoni-ordóñez *et al*., 2018; Ortiz-sánchez *et al*.,

2016). Hence, these biomarkers could potentially serve as therapeutic targets for cervical cancer.

Notch signalling is a type of direct intercellular communication that plays a crucial role in regulating the stem cell proliferation, cell fate determination, and apoptosis during embryonic development. Presently, it is apparent that the Notch signalling is significantly involved in the progression and onset of cancer. Notch1, a member of the Notch family, has been implicated in numerous types of cancer, including cervical cancer. It has been demonstrated to tightly connect with multiple signalling pathways that are involved in tumorigenesis (Gharaibeh *et al.,* 2020). Emerging evidence suggests that Notch1 signalling plays crucial roles in regulating the proliferation, self-renewal, differentiation, tumorigenicity, and migration of CSCs. Studies have found that Notch1 is overexpressed in CSCs, and inhibiting this pathway causes CSCs to lose their stemness characteristics. Thus, targeting the Notch1 signalling offers an additional therapeutic approach that can employed for the management of cancer with high levels of Notch1 (Maliekal *et al*., 2008; Xiao *et al*., 2017).

Natural products have been increasingly documented for their efficacy in cancer chemoprevention and therapeutics. The potential of medicinal plants and their bioactive compounds to address the growing demand for alternative, biocompatible, eco-friendly, safe, and cost-effective anticancer therapy has drawn significant interest among researchers (Gezici & Şekeroğlu*,* 2019; Greenwell & Rahman*,* 2015). Numerous secondary metabolites derived from plants, including terpenes, nitrogen-containing compounds, and phenolic compounds, have demonstrated anticancer properties against diverse types of cancers. These effects are attributed to multiple mechanisms, such as the inhibition of cancer-triggering enzymes and hormones, the

activation of DNA repair mechanisms, the induction of antioxidant activity, the augmentation of protective enzyme production, and the enhancement of the immune system (Shukla *et al*., 2015).

Clinacanthus nutans (*C. nutans*), a tropical herb belonging to the Acanthaceae family, has been extensively used as herbal medicine in Malaysia, Indonesia, Thailand, and China for treating a variety of ailments, including skin rashes, insect bites, snake bites, diabetes, and gout. Its medicinal value is further supported by a number of experimental studies that reported high anti-inflammatory, antiviral, antioxidant, and anti-diabetic activities in this plant species (Alam *et al.,* 2016; Shim *et al*., 2014; Yahaya *et al*., 2015). At the moment, this plant has been widely studied for its potential efficacy in cancer therapy. It has been demonstrated that *C. nutans* possesses potent cytotoxic and inhibitory effects on several cancer cells, including liver, lungs, breast, brain, and cervical cancers (Huang *et al*., 2015; Ng *et al*., 2017; Yong *et al*., 2013; Yusmazura *et al*., 2017). SF1, a fraction from *C. nutans* leaf extract, has been identified as one of the compounds responsible for the anticancer properties of *C. nutans*. Previous studies have demonstrated that SF1 exhibits significant and selective cytotoxic effects on cervical cancer cell lines, indicating its ability to specifically target and eliminate cancer cells whilst leaving normal healthy cells unaffected (Roslan *et al.,* 2018; Zainuddin *et al.,* 2019). It is widely acknowledged that the primary drawback of conventional anticancer therapy is its lack of selectivity for cancer cells, which can lead to numerous adverse complications (Villela-Martinez *et al*., 2017). Therefore, the selective cytotoxicity of SF1 towards cancer cells renders it a potentially efficacious and safe anticancer agent. Given the critical role that CSCs play in promoting the growth and progression of cancer, additional research is warranted to elucidate the effectiveness of this natural compound in eradicating CSCs.

1.2 Problem statement

Despite the advances in cervical cancer prevention and diagnosis, the disease remains prevalent among women globally. Recent reports have highlighted a rise in the incidence of advanced cervical cancer, which is correlated with poor treatment outcomes (Kumar *et al*., 2023). The challenges that arise in the management of cervical cancer, including cancer recurrence, metastasis, and multiple drug resistance, have been explained by the CSC theory. Existing cancer therapies are insufficient to eliminate the CSCs, owing to the unique properties and extensive array of survival mechanisms employed by the cells (Di Fiore *et al*., 2022; Huang & Rofstad, 2017). Until now, functional anticancer regimens for targeting the cell population have yet to be established. One of the major challenges in targeting the CSC population is the lack of comprehension of the specific biomarkers and molecular pathways involved in their tumorigenesis, which warrant rigorous investigations (Agliano *et al*., 2017).

Synthetic chemotherapeutic drugs are well-known for their detrimental side effects, and the process of discovering and developing them for clinical use presents multiple hurdles and requires significant financial and time commitments (Junqueira & Chammas, 2018). Thus, naturally occurring phytochemicals have emerged as intriguing alternatives to synthetic chemotherapy drugs. Natural products have been indicated to be more effective, have fewer side effects, and are capable of targeting multiple cancer pathways (Cragg & Pezzuto, 2016; Rahman, 2016). Nevertheless, reports on the inhibitory effects of natural or dietary compounds on CSCs were scarce, as most studies only concentrated on normal cancerous cells. This includes *C. nutans*, which has been documented to possess anticancer effects against numerous cancer types, including cervical cancer, yet no studies have been done to test its efficacy against CSCs.

1.3 Rationale of study

To the best of our knowledge, this is the first study into the effects of *C. nutans* on the CSC population. Our research colleagues previously identified SF1, a semi-purified fraction of *C. nutans*, which demonstrated potent anticancer properties against cervical cancer cell lines (Zainuddin *et al*., 2019, 2020). In light of the critical role that CSCs play in promoting cancer growth and progression, the purpose of this investigation was to further elucidate the capacity of SF1 to eliminate CSC population derived from cervical cancer.

The findings from this study could contribute to a deeper understanding of the biology of cervical CSCs (CCSCs), and offer novel strategies to eradicate the cells by modulating their stemness biomarkers and signalling pathways. In addition, the ability of SF1 to inhibit CCSCs could provide additional evidence of the compound's efficacy and support the rationale for further preclinical and clinical trials for development of SF1 as a prospective anticancer agent for eliminating both normal cancerous cells and CSCs in cervical cancer. In the long run, the success of this study could potentially enhance cervical cancer management, support long-term cancer recovery, and boost the quality of life for cancer survivors. Additionally, this study could expand the existing body of literature on the potential of natural compounds to combat cancer, with an emphasis on CSCs. Besides, this work may also draw attention to the significant value of local medicinal plants as a source of potent anticancer drugs.

1.4 Research objectives

1.4.1 Main objective

The main objective of the study was to elucidate the potential of SF1, a standardised fraction from *C. nutans* leaves, as a promising therapeutic agent for cervical cancer by targeting the CCSCs through the inhibition of their stemness properties and the signalling pathway involved in their self-renewal and tumorigenicity.

1.4.2 Specific objectives

1) To determine the cytotoxicity effect of SF1 on cervical cancer stem-like cells using a cell viability assay and sphere formation assay.
2) To analyse the effect of SF1 on the expression of selected stemness markers, including CD49f, CK17, Sox2, Nanog, and Oct4, in cervical cancer stem-like cells using a flow cytometry assay.
3) To examine the effect of SF1 on the *in vivo* tumorigenicity of cervical cancer stem-like cells using nude mouse xenograft models.
4) To evaluate the effect of SF1 on Notch1 expression in cervical cancer stem-like cells *in vitro* via Western blotting and quantitative RT-qPCR, and *in vivo* using immunohistochemistry assays.

1.5 Research hypothesis

1.5.1 General hypothesis

This study hypothesises that SF1 can target CCSCs by inhibiting their stemness properties through the attenuation of NOTCH1 signalling.

1.5.2 Specific hypothesis

1) SF1 can reduce the viability and sphere-forming ability of cervical cancer stem-like cells.
2) SF1 can inhibit the expression of stemness markers including CD49f, CK17, Sox2, Nanog, and Oct4, in cervical cancer stem-like cells.
3) SF1 can suppress the tumorigenicity of cervical cancer stem-like cells when xenografted into nude mice.
4) SF1 can downregulate the Notch1 protein and gene expression in cervical cancer stem-like cells.

CHAPTER 2

LITERATURE REVIEW

2.1 Cancer

Cancer is a disease that results from uncontrolled proliferation and expansion of cells. Almost 90 percent of all cancer aetiologies are linked to environmental factors, including lifestyle factors, infection, and radiation, while another 10 percent are genetically derived. Cancer development is a multistage process involving cellular and molecular events that induce the pathologic transformation of a normal cell into a neoplastic cell (Hursting *et al*., 1999; Klaunig, 2019). Cancer cells can be differentiated from healthy cells by their distinct characteristics, including sustaining proliferative signalling, avoiding growth suppressors and death, invasiveness and metastatic ability, inducing angiogenesis, promoting tumour inflammation, and avoiding immune destruction (Xu & Mao, 2016).

In recent years, there has been a decline in mortality rates for many types of cancer due to advancements in early detection and intervention measures. Despite this progress, cancer remains the second-leading cause of death globally, after cardiovascular disease (Obafemi & Omahi-Ottah, 2023). According to the World Health Organisation (WHO), cancer is the major cause of death in the population below the age of 70 years old in most countries in the world (WHO, 2021). In 2020, GLOBOCAN reported that cancer caused approximately 19 million new cases and 10 million deaths worldwide, accounting for one in every six deaths, and these estimates are expected to double by 2040. Women are most commonly affected by breast, colorectal, lung, cervical, and thyroid cancer, while men are most commonly affected by lung, prostate, colorectal, stomach, and liver cancer (Sung *et al*., 2021).

Furthermore, the recent COVID-19 pandemic has resulted in catastrophic changes to cancer care, including limited access to health-care systems, the suspension of screening programs, and the delays in diagnostic and treatment. If not addressed promptly, these problems may worsen the patient's morbidity and mortality in the future (Patt *et al*., 2020).

Surgery, radiation therapy, and chemotherapy have been the primary treatments for cancer for decades. Chemotherapy is a widely used cancer treatment modality that can be used alone or in combination with radiotherapy. These drugs induce apoptosis and necrosis in malignant cells by causing DNA damage, cell cycle arrest, oxidative stress, and cytoskeleton disruption. Hormonal therapy is also commonly used, particularly for oestrogen and progesterone receptor-positive cancers like breast and prostate cancer. It slows tumour growth by blocking oestrogen binding to oestrogen receptors and androgen-to-oestrogen conversion. Nevertheless, the outcomes of these conventional treatments are frequently hampered by the development of multidrug resistance and adverse side effects, as well as other limitations such as lack of specificity, inappropriate biodistribution, and a poor pharmacokinetic profile (Abraham & Staffurth, 2016; Miller *et al*., 2016; Palumbo *et al*., 2013).

Thus, modern cancer research has primarily focused on developing treatment modalities with the highest response rate and minimal side effects. Nowadays, improved understanding of the molecular mechanisms underlying cancer has led to the advancement and evolution of cancer treatments. New approaches, including a combination of drugs, targeted therapies, immunotherapy, stem cell therapies, nanoparticles, gene therapy, and natural antioxidants, are being used to enhance drug delivery and mitigate the development of drug resistance. However, the therapeutic

level has yet to be reached that reduces mortality and prolongs survival time for advanced cancers. With the advent of new genomic and molecular medicines, the construction and precise interpretation of molecular tumour profiles have become increasingly important in identifying potential targets for effective anticancer drug delivery. (Debela *et al*., 2021; Dede *et al*., 2023).

2.2 Cervical cancer

2.2.1 Anatomy of cervix

The cervix is a fibromuscular organ that forms a canal between the vagina and the uterus. It is cylindrical in form, measuring approximately 4 cm in length and 3 cm in diameter, and consists of opposing anterior and posterior walls. It consists of the internal OS (the opening between the cervix and the uterus), the endocervix (the inner part of the cervix that forms the endocervical canal), the ectocervix (the outer part of the cervix that opens into the vagina), and the external OS (the opening between the cervix and vagina). The region between the endocervix and ectocervix is described as the squamocolumnar junction. The cervix receives its arterial blood supply from the internal iliac arteries, and its venous blood drains into the hypogastric venous plexus. The cervix's lymphatic vessels are derived from the common iliac, external iliac, internal iliac, obturator, and parametrial lymph nodes. The cervix receives its nerve supply from the hypogastric plexus (Prendiville & Sankaranarayanan, 2017; Britannica, 2023).

The inner and outer parts of the cervix are lined by distinct types of cells. The endocervical canal is lined by columnar (glandular) epithelium, consisting of a single layer of tall cells with darkly stained nuclei close to the basement membrane. This

columnar epithelium is organised into numerous folds and invaginations within the cervical stroma to produce endocervical crypts or glands. Whereas the ectocervix is lined by squamous epithelium that is uniform, stratified, and non-keratinizing. A single layer of round basal cells with large dark-staining nuclei and little cytoplasm forms the lowest layer of the squamous epithelium. These basal cells proliferate and mature to form the subsequent layers of tissue. The squamocolumnar junction is the region where glandular epithelium transitions into squamous epithelium; therefore, it contains both columnar and squamous cells. This region is also known as the transformation zone, and its location relative to the external OS varies based on age, hormonal status, menstrual status, birth trauma, oral contraceptive use, and pregnancy. It is also the most common site of origin for cell abnormalities, metaplastic alterations, and cervical cancer (Prendiville & Sankaranarayanan, 2017; Jordan *et al.,* 2009).

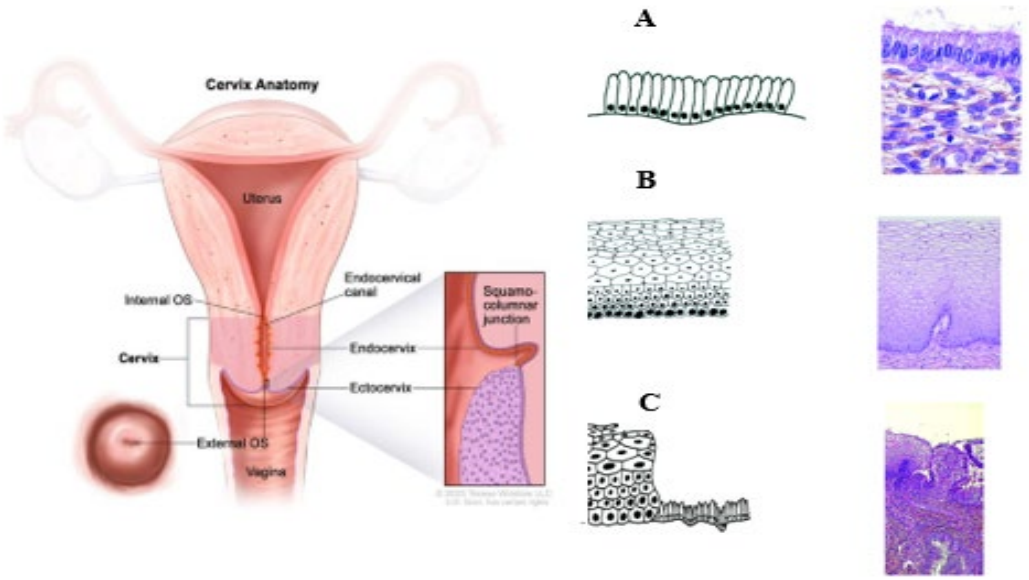

Figure 2.1 Anatomy of the cervix and the histological lining. (A) Columnar epithelium of the endocervical canal. (B) Squamous epithelium of the ectocervix. (C) The squamocolumnar junction (SCJ). Adapted from the National Cancer Institute, 2023 and Prendiville & Sankaranarayanan, 2017

2.2.2 Prevalence and risk factors of cervical cancer

Cervical cancer is one of the most common malignancies of the female reproductive organ. The two most common types of cervical cancer are adenocarcinoma (AC) and squamous cell carcinoma (SCC), which are classified based on their histological varieties. AC originates from the glandular cells and accounts for 25% of cervical cancer cases, whereas SCC arises from squamous cells lining the outer part of the cervix (ectocervix) and accounts for up to 70% of cervical cancer cases (Hull *et al*., 2020; Rositch *et al.,* 2022). Other less common types of cervical cancer include adenosquamous or mixed carcinoma, small cell or neuroendocrine carcinoma, serous papillary carcinoma, and clear cell carcinoma (Small *et al.,* 2017). Human papillomavirus (HPV) infection has been identified as the single most significant etiological agent of cervical cancer. Up to 99.7% of cervical cancer cases are caused by persistent infection with HPV 16 and 18, which are known as high-risk or oncogenic HPV subtypes. The correlation between human papillomavirus (HPV) and cervical cancer was first demonstrated in the early 1980s following the identification of the HPV 16 subtype in cervical cancer tissue by Harald zur Hausen (Burmeister *et al*., 2022). Apart from that, it is estimated that 1% of women in the general population, particularly those younger than 25 years, carry these high-risk HPVs (Zhang *et al.,* 2020).

Cervical cancer represents a global public health problem and economic burden, particularly in low- and middle-income countries. Despite the introduction of the Pap smear screening test and preventative vaccines, the prevalence of cervical cancer has continued to climb. In 2020, approximately 604,127 cases and 341,831 deaths related to cervical cancer were recorded globally, which correspond to 13.3 cases per 100,000 women and 7.2 deaths per 100,000 women, respectively.

Furthermore, there is a clear socioeconomic gradient in cervical cancer occurrences, with underdeveloped and developing countries having a three times higher incidence rate and a six times higher mortality rate than developed countries, which offer better and higher-quality screening, prompt treatment, and routine follow-up care. Whereas in Malaysia, approximately 1,700 cases of cervical cancer and 1000 deaths from the disease are reported annually, making it the fourth most prevalent cancer in women and the second most common cancer among women aged 15 to 44. Overall, these statistics are still far from the goal set by the WHO Global Cervical Cancer Elimination Initiative in 2020, which intended to reduce the incidence of cervical cancer to below 4 cases per 100,000 women in every country and to narrow the global disparities in the incidence of cervical cancer. (International Agency for Research on Cancer (IARC), 2023; Singh *et al*., 2023).

Several risk factors have been linked to persistent HPV infection and cervical cancer progression, including sexual, reproductive, environmental, lifestyles, and genetic factors. High-risk HPV infection is typically transmitted via sexual intercourse, leading to squamous intraepithelial lesions that spontaneously regress after 6 to 12 months. However, in a small percentage of cases, the lesions persist and develop into cancer. Besides, few studies have demonstrated that other sexually transmitted infections, such as chlamydia trachomatis, herpes, and human immunodeficiency virus (HIV), can raise the risk of cervical squamous cell carcinoma by suppressing the immune system and increasing the host's vulnerability to HPV. An increased risk of cervical cancer is also observed in women with multiple sexual partners, early age at first intercourse, multiparity, and prolonged oral contraceptive pill (OCP) consumption. Furthermore, smoking and obesity are found to double the risk of developing cervical cancer, most likely due to the induction of immune

suppression and hormonal imbalance, respectively (Momenimovahed & Salehiniya, 2017; Zhang *et al*., 2020). Additionally, few studies have revealed the association between inherited genetic predispositions, such as genetic polymorphisms in the tumour suppressor gene (TP53), and cervical cancer risk (Alsbeih *et al*., 2013).

2.2.3 Pathogenesis of cervical cancer

Persistent high-risk HPV 16/18 infection of the cervix, particularly near the squamocolumnar junction, can lead to the development of precancerous lesions and subsequent cancerous transformation of cells. In brief, the presence of wounds or abrasions in the cervical epithelial layer facilitates the infiltration of the human papillomavirus (HPV) into the basal layer of the epithelium, leading to cellular invasion. Upon initial contact with the basal cells, the human papillomavirus (HPV) is capable of traversing the nuclear membrane, thereby gaining access to the cell nucleus and subsequently integrating itself into the genome of the host cell. Indeed, the presence of viral integration was evident in 80% of HPV 16- and 100% of HPV 18- positive cervical cancer. The integration of viral DNA into the host genome results in the production of several oncoproteins, including E6 and E7, which exploit the cellular machinery of the host cell to induce and maintain the cervical cancer phenotypes (Balasubramaniam *et al*., 2019; Gómez & Santos, 2007).

The infected cervical epithelial cells will undergo a transformation process from a state of well-organised cellular structure to high levels of dysplasia. The precancerous lesions of the cervix are referred to as cervical intraepithelial neoplasia (CIN). CIN is classified histologically based on the extent of dysplastic changes. CIN1 is distinguished by the presence of mild dysplasia, evidenced by the presence of koilocytes (cells exhibiting enlarged and irregular nuclei together with a perinuclear

halo), binucleate cells, and dyskeratotic cells. CIN2 refers to the presence of moderately heterogeneous lesions that affect two-thirds of the epithelium. While CIN3 is characterised by the presence of severe dysplasia that affects more than two-thirds of the epithelium. CIN2 and CIN3 are also classified as high-grade squamous intraepithelial lesion (HSIL) (Balasubramanian *et al*., 2019). It has been reported that approximately 10% of CIN lesions subsequently progress to carcinoma in situ and invasive carcinoma after 10 to 30 years when left untreated or if other risk factors are incorporated (Alsbeih *et al*., 2013).

At the invasive stage of cervical cancer, cancer cells have disseminated from the surface of the cervix to the underlying cervical tissue or other organs. According to the International Federation of Gynaecology and Obstetrics (FIGO) revised staging of cervical cancer (2018), invasive cervical cancer is classified into four primary stages: stage I, II, III, and IV. Stage I denotes the presence of cervical cancer that is strictly confined to the cervix. Conversely, stage II signifies the spread of cancer beyond the uterus but not extending onto the lower third of the vagina or to the pelvic wall. Stage III refers to cancer that has extended to 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. Whereas stage IV indicates the presence of cancer metastases that have extended beyond the true pelvis to involve adjacent pelvic organs and/or distant organs. Invasive cervical cancer is associated with a poor prognosis necessitating the implementation of comprehensive treatment measures (Batla *et al*., 2021; Burmeister *et al.,* 2022).

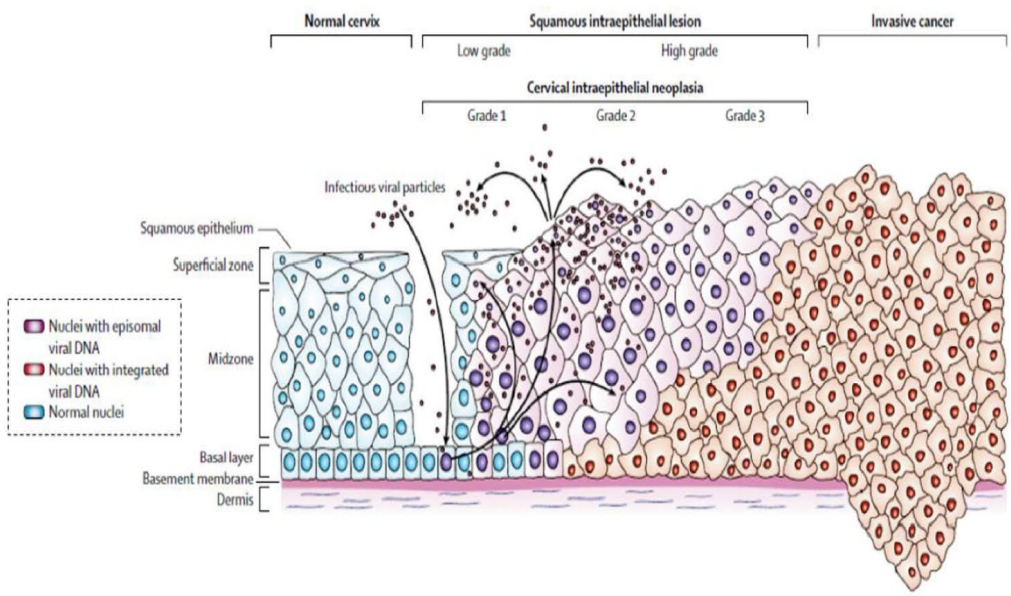

Figure 2.2 Depiction of HPV infection and cervical cancer development. Adapted from Burmeister *et al*., 2022

2.2.4 Existing therapeutic approaches and drawbacks

The conventional treatment approach for cervical cancer encompasses surgical intervention, radiation therapy, and chemotherapy, alone or in combination. The determination of an appropriate treatment modality typically relies on various aspects, such as the stage of the cancer, the size of the tumour, the patient's age, and underlying comorbidities (Colombo *et al*., 2012). In the case of precancerous lesions, minor surgical procedures, such as cryosurgery, thermal ablation, and laser surgery, are used to excise and remove the lesions. In contrast, the management of invasive cancer necessitates a more extensive, radical, and complex combination of treatments, including conization, hysterectomy, trachelectomy, irradiation, and/or chemoradiation (Burmeister *et al.,* 2022).

Radiation therapy may be administered in two distinct contexts: as a neoadjuvant treatment preceding surgery to reduce the tumour size, or as an adjuvant

treatment following surgery to eradicate any residual tumour cells. In chemotherapy, platinum-based drugs, including cisplatin, carboplatin, paclitaxel, and topotecan, are commonly used as the standard treatment for advanced and recurrent cervical cancer. Cisplatin serves as the first-line drug for concurrent chemoradiation therapy (CRT), as it is known to sensitise the cells to radiation. Additionally, new cervical cancer treatments like immunotherapy and targeted medicines are currently undergoing clinical trials. Cancer immunotherapy, such as immune checkpoint inhibitors (ICIs), demonstrates anticancer properties through the augmentation of the patient's immune system. While targeted treatments, like bevacizumab, have been shown to inhibit tumour growth by blocking aberrant signalling pathways (George *et al.,* 2022).

Despite advancements in treatment modalities, a substantial proportion of patients exhibit recurrence, resistance to chemotherapy and/or radiation, and metastases following treatment (Zhang *et al*., 2012). Recurrent cervical cancer refers to the local re-growth of tumours or the development of lymph node or distant metastases at least six months following the regression of the primary tumours. Typically, one-third of cervical cancer will recur within two years of initial treatment. Following surgery and CRT, recurrence rates were 27% and 32%, respectively. While some studies reported that the incidence of cervical cancer recurrence is 11%-22% for stages I-II and 28%-64% for stages III-IV. Other reports indicate that distant recurrence is the predominant pattern of relapse observed in patients with locally advanced cervical cancer who receive CRT. Recurrent cervical cancer has been correlated with an unfavourable prognosis, as evidenced by a global one-year survival rate of less than 30% among patients with advanced or recurrent disease (George *et al.*, 2022; Miccò *et al*., 2022).

Persistent or recurrent cervical cancer is mainly attributed to chemotherapy or chemoradiation failures and the emergence of treatment resistance. In studies done over the past 40 years, the response rates to cisplatin have been found to be between 18% and 50% for doses between 50 mg/m^2 and 100 mg/m^2 every 3 weeks. While more recent studies have demonstrated that the overall response rate to cisplatin was only 30% in patients with stage IVB, recurrent, or persistent cervical cancer. In addition, it was reported that only six out of 149 patients who received cisplatin-based treatment showed a complete response. Thus, low response rates and resistance remain an ongoing and unsolved concern in clinical settings (Masadah *et al*., 2021). Furthermore, patients undergoing chemotherapy or radiotherapy often encounter a diverse array of side effects, including nausea, alopecia, anaemia, neurotoxicity, non-specific tissue damage, and neutropenia. This is due to the lack of specificity of conventional treatments, which can cross-react with the growth signals of normal cells and disrupt the cell growth and function (Mahato *et al.,* 2011).

2.3 Cervical cancer stem cells

2.3.1 Cancer stem cells theory

Cancers are characterised as tissues made up of a heterogeneous population of cells with a range of biological characteristics and the capacity to self-renew. The majority of cells in bulk tumours are non-tumorigenic, have a limited ability to self-renew and die after transient differentiation. CSC theory postulates that only a small subset of cancer cells possess indefinite self-renewal, proliferation and differentiation potential to develop into any cell in the malignant population and drive oncogenesis. (Han *et al.,* 2013). The concept of cancer evolution from a small population of cells with stem cell properties has been proposed since the past 150 years (Kreso & Dick,

2014). The advances in stem cell biology, the emergence of detection technologies like cell sorting based on the different expressions of surface markers, and the development of immunocompromised animal models have facilitated researchers in investigating the CSC properties and validating the CSC hypothesis (Cho & Clarke, 2008).

CSCs were first described in acute myeloid leukaemia, specifically as a subset of cells expressing the surface marker $CD34^{+}CD38^{-}$. These cells have demonstrated the capacity to undergo self-renewal, proliferation, and differentiation, as well as the ability to reconstitute heterogenous tumour growth when serially passaged in NOD/SCID (nonobese diabetic/severe combined immunodeficiency mice) mice (Bonnet & Dick,1997; Lapidot *et al*., 1994). Hence, the cells are labelled as leukemic stem cells (LSCs). This discovery has sparked extensive investigation into the existence of CSCs in solid tumours. The initial efforts to isolate and characterise CSCs from solid tumours were conducted in breast cancer. The study by Al-Hajj *et al.* (2003) discovered that breast cancer cells display varied surface protein expression. However, only a subset of cells expressing $CD44^{+}CD24^{-}$ were capable of initiating tumour growth in immunodeficient mice. Subsequent studies have also revealed the presence of CSCs in various types of tumours, including brain, pancreatic, liver, lung, colon, head/neck, melanoma, ovarian, and cervical cancers, which provide additional compelling support for the CSC theory (Lathia *et al*., 2020).

CSCs are widely defined as cells within a tumour that have the ability to self-renew and give rise to the heterogenous lineages of cancer cells that constitute the tumour (Bomken *et al.,* 2010). CSCs may divide symmetrically to produce two identical daughter CSCs or asymmetrically to produce one daughter CSC and one differentiated progenitor cell. In contrast to normal progenitor cells, cancer progenitor cells have a greater and prolonged proliferative capacity, resulting in the expansion of

cancer cells progeny and tumour growth (Fulawka *et al.,* 2014). Emerging studies have proposed that CSCs may originate from normal stem cells, progenitor cells, or more differentiated cells that undergo genomic instability or plasticity in response to oncogenic stimuli. This genetic and epigenetic instability may lead to the buildup of gene mutations that confer self-renewal and tumorigenic potential on the cells (Yu *et al*., 2012). CSCs share many similar characteristics to normal stem cells, including self-renewal and differentiation potential, specific surface markers and oncogene expression, common signalling pathways, apoptosis evasion, and the presence of stem cell niches. The primary distinction between the two cell types is in their tumorigenic potential, wherein CSCs possess the ability to induce tumour formation in xenograft animal models, whilst normal stem cells lack this capacity (Han *et al*., 2013).

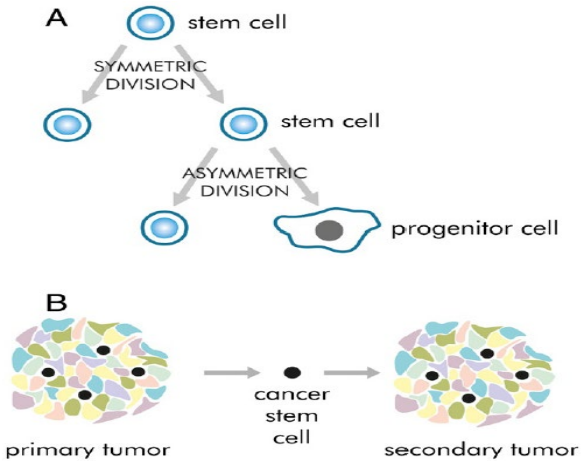

Figure 2.3 Two features defining cancer stem cells (A) Self-renewal capacity to produce offspring that retain stemness characteristics. (B) The ability to restore the heterogeneous cancer cell population. Adapted from Fulawka *et al*., 2014

It is becoming evident that CSCs are responsible for the development of resistance to cancer treatment, metastases and the recurrence of tumours. CSCs acquire the capacity to evade conventional chemotherapy and radiotherapy through several mechanisms, such as stem cell quiescence, microenvironment protection, drug efflux pump activation, and upregulation of anti-apoptotic and DNA repair pathways (Batlle

& Clevers, 2017). In addition, CSCs possess the migratory and invasive capacities to promote cancer metastases via the epithelial-mesenchymal transition (EMT). EMT is a sequential process that triggers the transformation of malignant epithelial cells into more mesenchymal and motile cells and induces the loss of cell-cell attachment, thereby allowing the cells to migrate, disseminate, and invade distant organs (Agliano *et al.,* 2017). In this regard, the development of effective and comprehensive regimens to eradicate both CSCs and the bulk of disease is critical in order to achieve a complete cancer remission and improve patient survival rates.

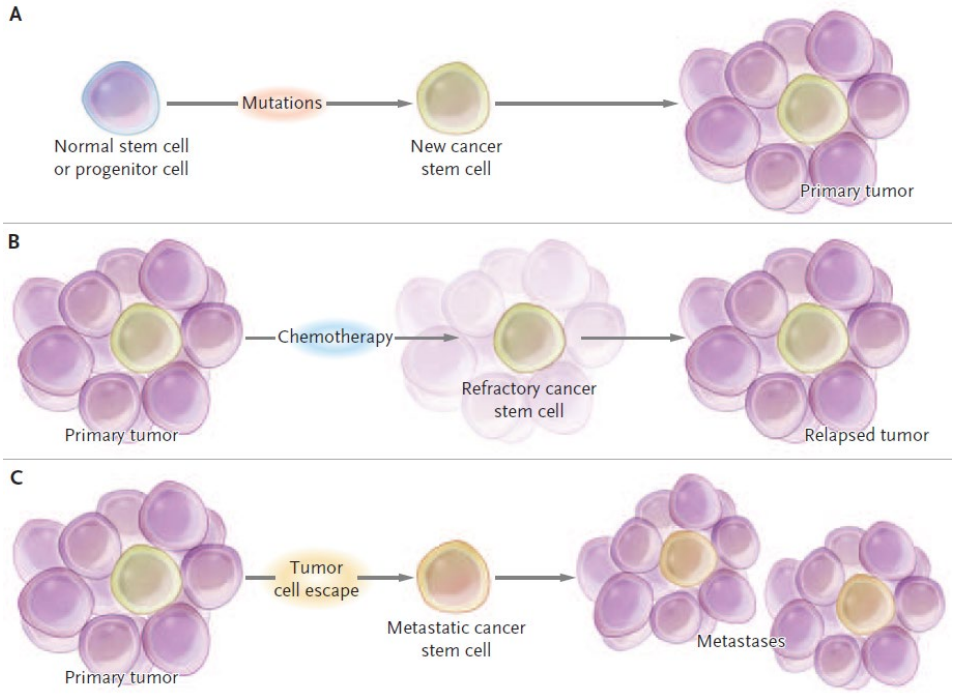

Figure 2.4 Role of cancer stem cells in tumour formation and progression. Adapted from Jordan *et al.,* 2006

2.3.2 Role of cancer stem cells in cervical cancer

A number of clinical and experimental observations have confirmed the existence of CSCs in cervical cancer. It was hypothesised that the transformation zone of the cervical epithelium contains a niche of cells with a distinctive expression profile and embryonic characteristics. This group of cells refers to the basal or cuboidal

epithelial cells, which are considered the stem cells of the cervical epithelium which are the target of the HR-HPV infection (Sudhalkar *et al.*, 2019). The high expression of certain markers, such as CD44, CD49f, CK17, and CD133, on the cell membrane of the cells is known to attract HR-HPV binding to the receptors and promote viral internalization. Viral DNA is then released and transported to the cell nucleus, where the synthesis of viral oncoproteins E6 and E7 commences. The oncoproteins subsequently induce the inactivation of the endogenous tumour suppressor proteins, retinoblastoma-associated protein (pRb) and p53. This inactivation, in turn, leads to the overexpression of stemness-related genes, specifically Sox2, Oct4, and Nanog. Subsequently, these events result in inhibition of cell apoptosis, proliferation of the infected stem cells and promotion of tumour development (Organista-Nava *et al*., 2014, 2019).

Moreover, Sox2, Oct4, and Nanog have been associated with the maintenance of the cervical cancer stem cell (CCSC) population and the enhancement of CCSCs' self-renewal capacity. It was demonstrated that these stemness-related genes contribute to clonogenicity, proliferation, invasiveness, and drug resistance in CCSCs, which were achieved through the activation of ATP-binding cassette (ABC) transporters, ALDH1, and Musashi-1 (MSI1) gene expression (Organista-Nava *et al.,* 2014, 2019). Furthermore, Wang *et al*. (2014) reported that CCSC were resistant to cisplatin and epirubicin, and exhibited a higher invasive potential compared to the parental cervical cancer cells (HeLa). Additionally, it is evident that CSCs contribute to the presence of heterogeneous populations of carcinoma cells in cervical cancer due to their unique capability to undergo trans differentiation into vascular endothelial cells and tumour-associated stromal cells (Di Fiore *et al*., 2022). Hence, the primary focus

in the development of new therapies for cervical cancer should be on targeting the CSC population.

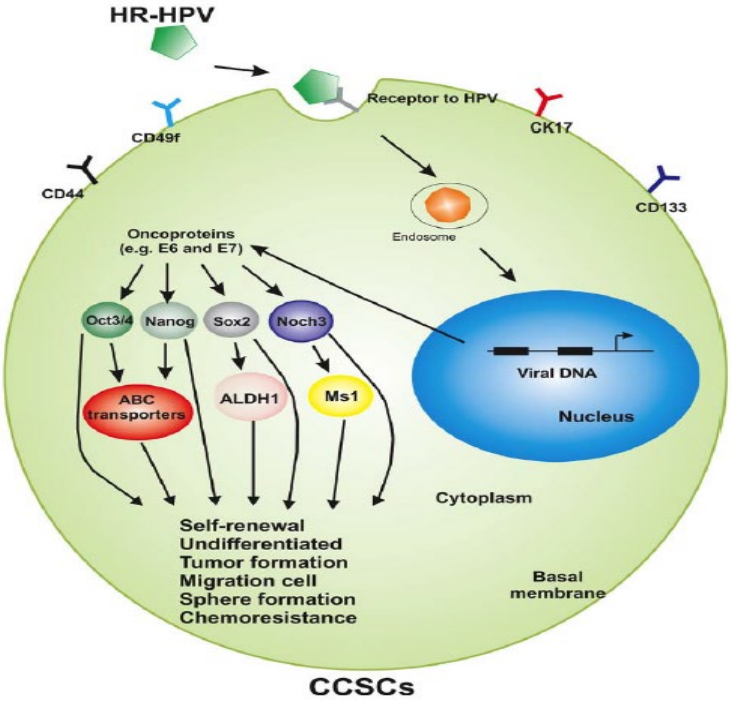

Figure 2.5 The regulation of cervical cancer stem cells (CCSCs) through the network mediated by the high-risk human papillomavirus (HR-HPV) oncoprotein. Adapted from Organista-Nava *et al*., 2019

2.3.3 Biomarkers for cervical cancer stem cells

The identification and isolation of CSCs from cervical cancer are usually performed using experimental approaches that involve the sorting of tumour cell populations, identifying surface markers that are differentially expressed in the CSC subpopulation, and assessing their tumorigenic capacity in immunodeficient mouse models. Currently, there is a growing number of stem cell markers for CCSCs, and researchers are continuously identifying novel markers. Several potential cervical epithelial stem cell markers, such as CD49f, CK17, Sox2, Nanog, and Oct4, have been used to identify and characterise CCSCs. These markers have been linked to poor prognosis and multidrug resistance in cancer patients and hence serve as potential