Gamma-secretase inhibitor IX (GSI) impairs concomitant activation of Notch and wnt-beta-catenin pathways in $CD44^+$ gastric cancer

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2. Müller A*, **Barat S***, Chen X, Bui KC, Bozko P, Malek NP.Comparative study of antitumor effects of bromelain and papain in human cholangiocarcinoma cell lines.Int J Oncol. 2016 May;48(5):2025-34. (* equal contribution)

3. Wutka A, Palagani V, **Barat S**, Chen X, El Khatib M, Götze J et al.,Capsaicin treatment attenuates cholangiocarcinoma carcinogenesis.PLoS One. 2014 Apr 18;9(4)

4. **Barat S**, Chen X, Bui KC, Bozko P, Götze J, Christgen M, Krech T, Malek NP, Plentz RR . Gamma-secretase inhibitor IX (GSI) impairs concomitant activation of Notch and wnt-beta-catenin pathways in CD44+ gastric cancer (Stem cell translational medicine; in press).

Abbreviations

5-FU	5-fluorouracil
ABC	avidin-biotin-complex
APC	adenomatosis polyposis coli
bHLH	helix-loop-helix
CA19-9	carbohydrate antigen 19-9
CDK8	cyclin dependent kinase 8
CE	cytoplasmic extract
CK1	casein kinase 1
CLL	chronic lymphocytic leukaemia
CMML	chronic myelomonocytic leukaemia
COX2	cyclogenase 2
CSC	cancer stem cells
DAB	diaminobenzidine
DDL 1	delta like
DHH	desert hedgehog
DMSO	dimethylsulfoxide
DNA	deoxynucleic acid
EGFR	epidermal growth factor receptor
EMT	epithelial-mesenchymal-transition
Erk	extracellular signal regulated kinase
FACS	fluorescence activated cell sorting
FCS	fetal calf serum
GC	gastric cancer
GSI IX	gamma secretase inhibitor IX
GTP	guanosine-5'-triphosphate
H	hour
HA	hyaluronic acid
HCC	hepatocellular carcinoma
HER2	human epidermal receptor 2
Hes1	hairy and enhancer of spilt-1
HNPCC	hereditary nonpolyposis colorectal cancer
HuGE	human genome epidemiology
IHH	indian hedgehog

Kg	kilogram
MAML	mastermind like
Mg	milligram
MLH1	mutL homolog 1
MLH1	mutL homology 1
Mm	millimetre
MNC	mononucleocytes
MSH2	mut 2 protein homolog 2
MSH2	mut 2 protein homology 2
Muc1	Mucin 1
NE	nuclear extract
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NICD	notch intracellular domain
NSCLC	non small cell lung cancer
OCIF	osteoclastogenesis inhibitory factor
PARP	poly (ADP-ribose) polymerase
PBS	phosphate buffered saline
PCD	programmed cell death
PCR	polymerase chain reaction
PDAC	pancreatic ductal adenocarcinoma
PI	propidium iodide
PS	phosphatidylserine
PTEN	phosphatase and tensin homolog
RAC1	ras-related C3 botulinum toxin substrate 1
ROS1	reactive oxygen species 1
RT	room temperature
SD	standard deviations
SHH	sonic hedgehog
siRNA	small interfering RNA
STAT3	signal transducer and activator of transcription 3
T-ALL	lymphocytic leukaemia
TIC	tumor initiating cells
TMA	tissue microarray
UK	United Kingdom

USA	United States of America
V	variant
v	volume
vs	versus
WHO	World Health Organisation
WST	Watersoluble tetrazolium

Zusammenfassung

Zusammenfassung

Hintergrund & Ziele: Magenkrebs ist die zweithäufigste Ursache aller Krebstodesfälle weltweit. Fortgeschrittene Stadien von Magenkrebs sind oft verbunden mit limitierter und geringfügiger chemotherapeutischer Behandlungsweise. Dieser Fakt hebt den Bedarf an neuen Fortschritten in der Behandlung von Magenkrebs hervor. Krebsstammzellen (CSCs) sind bekannt als einer der verantwortlichen Hauptkandidaten für Tumorrezidive, Metastasen und Resistenz gegen Krebstherapien in verschiedenen Arten von Magenkrebs. Studien haben gezeigt, dass Notch- und wnt-beta-catenin-Signalwege äußerst entscheidend für die CSC-Entwicklung sind. In dieser vorliegenden Studie haben wir uns auf die einhergehende Aktivierung von Notch- und wnt-beta-catenin-Signalwege in $CD44^+$ Magenkrebs beschäftigt. Wir haben ausserdem eine effektive Inhibierung von Notch- und wnt-beta-catenin-Signalwegen in $CD44^+$ gastrischen tumorauslösenden Zellen durch Verwendung des γ-secretase Inhibitors IX (GSI IX) gezeigt.

Methoden: Um den Effekt von GSI IX auf tumorauslösende gastrische CD44+ Zellen zu bestimmen, wurde das Wachstum, die epitheliale Plastizität und die Tumorentwicklung nach GSI IX Behandlung bestimmt. Hierzu wurden *in vitro*-Analysen zur Proliferation, Wundheilung, Invasion, Adhäsion und Tumorsphere-Bildung mit der gastrischen Tumorzelllinie MKN45 durchgeführt, um das Migrations-, Invasions-, Adhäsions- und Tumorpotenzial zu untersuchen.Mittels Western Blot wurden Notch- und Wnt-beta-catenin auf auf die Aktivierung der Signaltransduktionskette nach gamma-secretase Inhibitor IX (GSI IX) Behandlung getestet.Immunhistochemie, Immunfluoreszenz und durchflusszytometrische Analysen wurden genutzt um die $CD44^-$ und Hes1-Expression in humanem Gewebe, Zellen und Blut von Magenkrebs zu bestimmen. Für *in vivo*-Analysen wurden mittels Durchflusszytometrie isolierte $CD44^+$ Zellen subkutan in Nacktmäuse (NMRI-nu/nu) injiziert und mit GSI IX, oder PBS als Kontrolle, behandelt.

Ergebnisse: Die Behandlung mit GSI IX inhibiert effektiv das Zellwachstum, Migration, Invasion, Adhäsion und induziert Apoptose in $CD44^+$ gastrischen tumorauslösenden Zellen. Außerdem wurde eine komplette Inhibierung der Notch- und beta-catenin-nachgeschalteten Signalzielen beobachtet. Magenkrebspatienten mit einer Expression von CD44 und Hes1 zeigten allumfassend eine geringere

Überlebensrate. $CD44^+$ CSCs zeigten eine hohe Expression von Hes1 im Vergleich zu der $CD44^-$ Subpopulation. Interessanterweise ist unter allen Notch rezeptoren Notch1 enorm wichtig in der Vermittlung einer Interaktion zwischen Notch- und wnt-beta-catenin-Signalkaskaden in $CD44^+$ CSCs. Eine Runterregulation von sowohl CD44 als auch Notch1 durch siRNA inhibierte nachgeschaltete Ziele und bestätigte die vorgeschlagene Hypothese, dass CD44 einer Interaktion der Signale in gastrischen Tumorzellen vermittelt. Die GSI IX-Behandlung inhibiert wirksam die einhergehende Aktivierung von beiden (Notch und wnt-beta-catenin) Signalwegen in Magenkrebs durch spezifisches Abzielen auf $CD44^+$ gastrischen CSCs.

Schlussfolgerung: Somit unterstützt unsere Studie das Vorhandensein von bislang identifizierten $CD44^+$ CSCs in humanen gastrischen Tumorzellen. Außerdem hebt unsere Studie die Aktivierung von Notch und beta-catenin in gastrischen tumorauslösenden $CD44^+$ Zellen und dessen wirksame Inhibierung nach einer GSI IX-Behandlung hervor. Zudem zeigte die Expression von CD44 und Hes1 doppelt positive Zellen eine starke Korrelation mit der gesamten Überlebensrate der Patienten. Dafür könnte GSI IX eine mögliche wirksame alternative Behandlungsmöglichkeit für Magenkrebspatienten sein.

Summary

Summary

Background & Aims: Gastric cancer is the second most common cause of cancer related death in the world. Advanced stages of gastric cancer are often associated with limited and marginal chemotherapeutic regimes. This fact further emphasises on the need of new advances in gastric cancer treatement. Cancer stem cells (CSCs) known to be one of the main candidates responsible for tumor recurrence, metastasis and resistance to cancer therapy are identified and charecterised in different types of gastric cancers. Studies have indicated that Notch and wnt-beta-catenin pathways are crucial for CSC development. Here in this study we higlight on the concomitant activation of Notch and wnt-beta-catenin pathway in $CD44^+$ gastric cancer. We also showed effective inhibition of Notch and wnt-beta-catenin pathway in $CD44^+$ gastric tumor initiating cells using the γ-secretase inhibitor IX (GSI IX)

Methods: We analysed the effect of GSI IX on growth, epithelial plasticity and tumorogenicity of gastric tumor intiating $CD44^+$ cells. We have used gastric cancer cell line MKN45 in our experiments. For *in vitro* analysis proliferation,wound healing, invasion, adhesion and tumorsphere assays were performed to analyse the migration ,invasive, adhesive and tumorogenic potential of $CD44^+$ sorted gastric tumor initaiting cells after GSI IX treatment. Western blot analysis of downstream signaling targets of Notch and wnt-beta-catenin were tested after gamma-secretase inhibitor IX (GSI IX) treatment. Immunohistochemistry, immunofluorescence and flowcytometric analysis were used to determine CD44 and Hes1 expression in human GC tissues, cells and blood. For in vivo analysis sorted $CD44^+$ cells were subcutaneously injected into the nude mice *(NMRI-nu/nu)* and were treated with vehicle or GSI IX.

Results: GSI IX treatment effectively inhibits cell growth, migration, invasion, adhesion and induced apoptosis in CD44+ gastric cancer tumor initiating cells. Moreover, complete inhbition of notch and beta catenin downstream signaling targets were observed. Gastric cancer patientswith expression of CD44 and Hes1 showed overall reduced survival. $CD44^{+}$CSCs showed high expression of Hes1 as compared to the $CD44^{-}$subpopulation. Interestingly, amongst all Notch receptors, Notch1 was found to be important in mediating a crosstalk between Notch and wnt-beta-catenin signaling cascades in $CD44^{+}$CSCs. Silencing of both CD44 and Notch1 by siRNA inhibited downstream targets and reconfirmed the proposed hypothesis of CD44 mediated signaling crosstalk in GC cells. GSI IX treatment effectively inhibits the concomitant activation of both (Notch and wnt-beta-catenin) pathways in gastric cancer via specific targeting of $CD44^{+}$ gastric CSCs.

Conclusion: Thus, our study supports the presence of previously indentified $CD44^{+}$ CSC in human gastric cancer cells. Our study also highlights the activation of Notch and beta catenin in gastric tumor initiating $CD44^{+}$ cells and its effective inhibiton after GSI IX treatment. Moreover expression of CD44 and Hes1double positive cells showed a strong correlation with overall patient survival. Therefore, GSI IX could be a possible effective, alternative, treatment option for gastric cancer patients.

Introduction

Introduction

1.1 Gastric Cancer

Gastric carcinogenesis is a multi-step process which is governed by several factors. Gastric cancer is categorized into mainly two types: intestinal and diffuse. Intestinal type of gastric cancer is mainly based on environmental factors like lifestyle, helicobacter pylori infection etc whereas diffuse type is often related to genetic abnormalities (Figure 1). Gastric cancer remains to be seventh most common cause of cancer related death worldwide mainly due to poor prognosis. Advances in molecular medicine are of urgent need not only to understand in detail the carcinogenic process involved in gastric cancer progression, but also to offer new potential approaches for diagnosis, treatment and better therapeutic intervention (1, 2, 3).

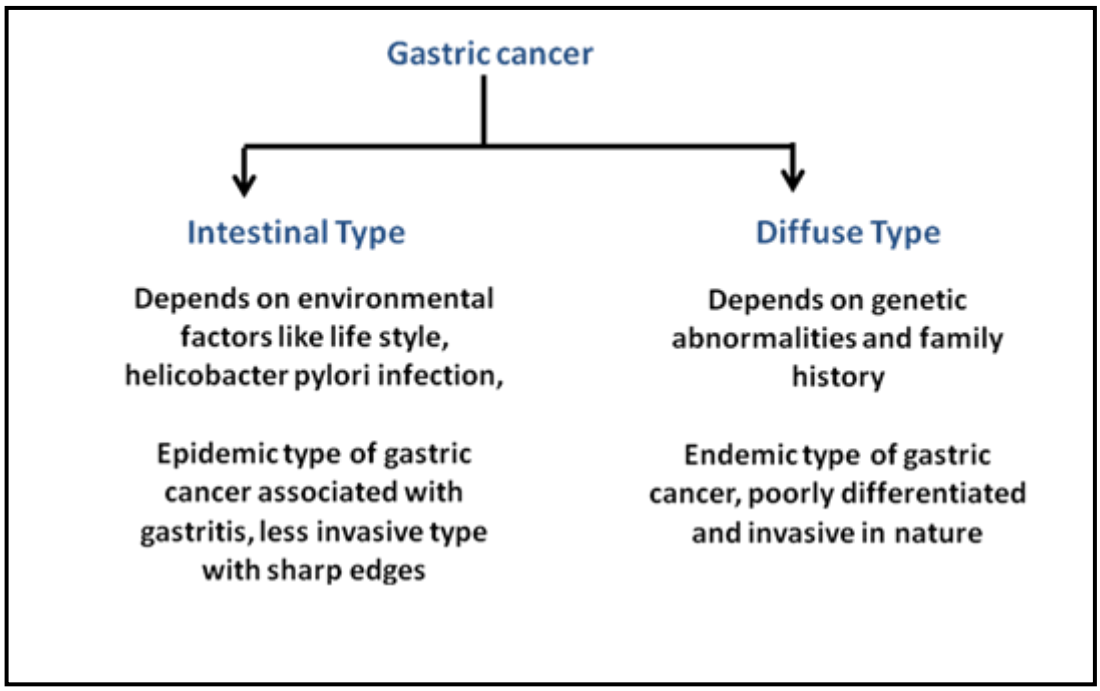


Figure 1: Gastric cancer classification. According to the Lauren classification gastric cancer is divided into two main types depending on the factors involved in gastric cancer development (Adapted from 1).

1.1.1 Incidence mortality and survival

Gastric cancer is one of the most common types of cancer prevalant worldwide and the third leading cause of cancer related death all over the globe. Interestingly, the global incidence rate of gastric cancer has declined rapidly over the past few decades mainly due to the identification of some of the common risk factors associated with gastric cancer progression. All though, the response rate of gastric cancer to multi-agent therapy is 50% or greater but the median survival rate is still limited. The five year survival rate of gastric cancer is less than 10% in western countries in comparison to Japan (over 50%). Moreover, limited patients can undergo curative surgery (3). The lack of curative therapeutic options makes gastric cancer as one of the most lethal and deadly disease for the mankind. In the United States approximately 22,220 patients are diagnosed annually with gastric cancer. China and Japan are the leading countries across the globe with a high rate of gastric cancer.Despite an overall decrease in gastric cancer incidence worldwide over the past few decades but interesting aspects such as age, sex etc. has been observed to play an important role in gastric cancer incidence. In China an increase in gastric cancer incidence has been observed in older patients as compared to younger once, a more remarkable difference has been observed between female, male genders. Notably, that age of onset of gastric cancer in China is younger compared to the west (4, 5).

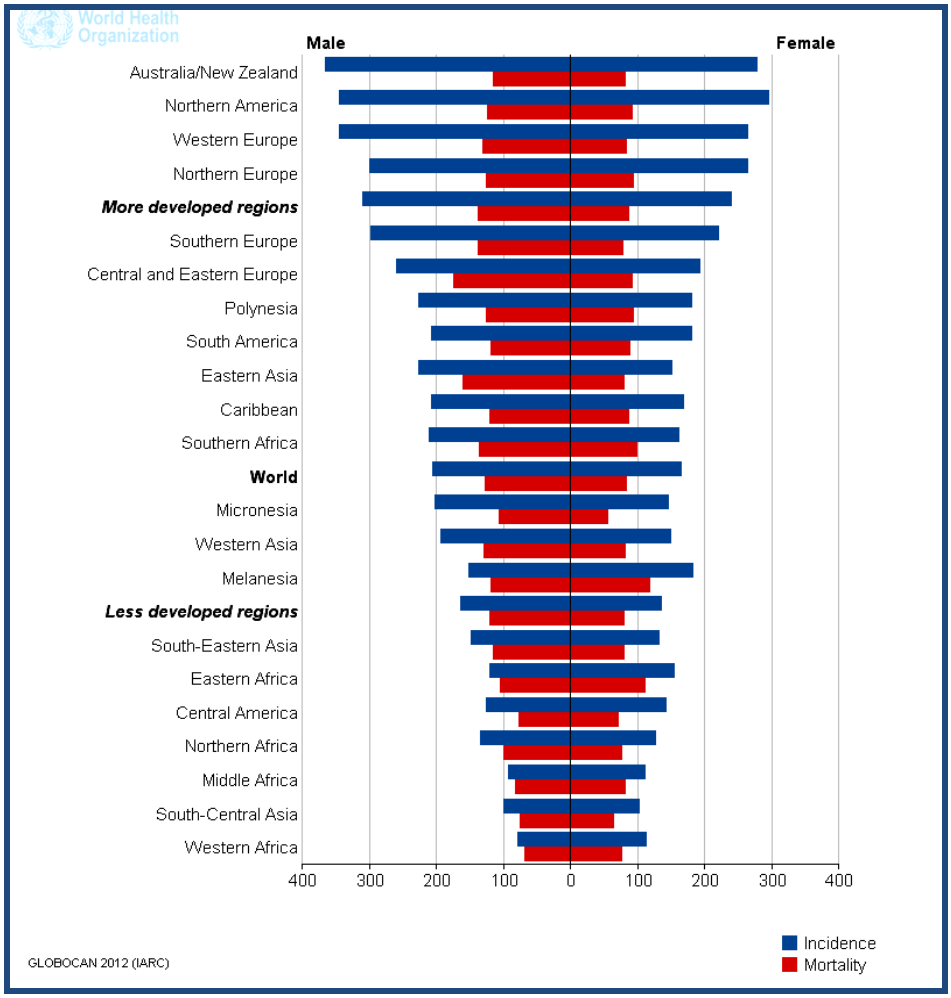


Figure 2: Worldwide incidence and mortality rate of gastric cancer patients depending on gender (Male vs. Female) (GLOBOCAN 2012)(Adapted from 6).

Geographical variations and migration studies has revealed an immense effect on the incidence rate of gastric cancer. Eastern Asia, Eastern Europe and South America has relatively higher incidence rate as compared to North America and parts of Africa (Figure 2). Importantly, a difference in incidence rate and mortality has been observed from North to South; Northern areas have a higher mortality rate compared to south. Such North/South divide for gastric cancer incidence and mortality has been observed in countries like Japan, England and China. Therefore, this fact highlights that geographical areas are associated with the incidence and mortality of gastric cancer. The histology pattern of gastric cancer is also changing with an increase in incidence of diffuse type of gastric cancer as compared to the intestinal type (7, 8, 9).

According to the GLOBOCAN database organized by the World Health Organisation (WHO) the country-specific mortality rate for gastric cancer reports an annual percentage change in gastric cancer mortality from 3 to 4 percent for the major European countries. Leading countries from other continents such as republic of Korea, Japan, Australia, United States and South America have shown an annual percentage change of 4.3%, 3.5%, 3.7%,3.6% and 2.6% respectively in mortality rate of gastric cancer (7, 8).

Despite the decline in the mortality rate of gastric cancer the absolute number of new cases every year is increasing mainly due to the aging of the world population. Furthermore due to unclear reasons the decrease in mortality rate has been replaced by an upward trend for gastric cancer onset in younger patients in recent years. Therefore, gastric cancer will continue to be one of the alarming causes of cancer related death worldwide even in the foreseeable future (4, 5, 9).

1.1.2 Symptoms and diagnosis of gastric cancer

The classical representation of patients with gastric cancer is often well perceived. The main symptoms involved in gastric cancer are abdominal pain, anorexia, weight loss, vomiting and signs of anaemia. Gastric cancer is also associated with haematemesis, dysphagia and abdominal swelling due to ascites (2, 10). Abdominal mass is often identified in gastric cancer due to the presence of primary tumor or liver metastasis. Usually the recognition of such evident symptoms is always identified at a later or advanced stage thus the likelihood of a successful curative resection is often obscure. The chances of curative options like chemo or radiotherapy are limited therefore; in the majority of cases the only mode of treatment available is palliative in nature. Unfortunatley identification of these symptoms at an early stage during the evolution of the disease could be subjected to early endoscopy and treatment. However, this doesnot appear to be feasible as patients with early stages of gastric cancer are either asymptomatic or dyspepsia (no report of upper abdominal pain). According to a statistical report out of 100 patients of gastric cancer in Japan only 66% were asymptomatic, 34% showed primary symptoms, 21% complained of discomfort and only 4 reported to have loss of appetite (2, 10, 11). Therefore, gastric cancer being the second most lethal form of cancer after lung cancer in the world the main reason for this appalling diagnosis can be attributed to the limitation of detection

of gastric cancer at an early stage. Unfortunately, the disease is always detected at a later stage of the evolution and has already metastasized (2, 10, 11).

1.1.3 Morphology of gastric cancer

The full story of gastric cancer still remains incomplete and is in evolution (2). Gastric cancer often markedly demonstrates heterogeneity at both architectural and cytological level with several histological elements. Over the past few decades gastric cancer has been histologically classified based on Laurens classification. According to Lauren's classification gastric cancer can be classified into two main types: intestinal type and diffuse type adenocarcinoma and also indeterminate type is often considered as uncommon variant.The 2010 WHO classification recognized four major histological patterns of gastric cancer which includes tubular, papillary, mucinous and poorly cohesive. Poorly cohesive gastric cancer also includes signet ring cell carcinoma and the uncommon variant (1, 2, 12).

Tubular adenocarcinoma is the most common histological type of gastric cancer. It mainly forms polyploidy with irregularly fused or branching tubules of various sizes.

Papillary adenocarcinoma is a common histological variant often seen in stages of early gastric cancer. It is mainly associated with liver metastasis with high lymph node involvement, specifically characterized by epithelial projections with a central fibrovascular core.

Mucinous adenocarcinoma accounts for 10% of gastric cancer. Histologically this it is mainly characterized by mucinous pools which form almost 50% of the tumor volume with occasional signet ring cells floating in these pools.

Signet ring cell carcinoma is composed of a mixture of signet ring cells and non-signet ring cells.These tumor cells form irregular lace like glandular structures accompanied by desmoplasia in the gastric wall with an ulcerated surface. Signet cell carcinoma and other poorly cohesive carcinomas at antroplyoric regions specifically shows propensity to invade duodenum via various submucosal routes. Therefore, special attention needs to pay to these routes during surgical resection. However, pseudo signet ring cells donot show any invasive pattern (4, 8, 11).

In addition to the above mentioned division the WHO classification also includes other uncommon histological terms such as adenosquamous carcinoma, squamous carcinoma, hepatoid adenocarcinoma etc (12).

1.1.4 Genetics of gastric cancer

During gastric carcinogenesis accumulation of genetic and molecular abnormalities occurs which includes inactivation of oncogenes, overexpression of growth factors, inactivation of tumor suppressor genes, DNA repair genes etc (2, 9, 12, 16).

Many genes involved in gastric carcinogenesis have been linked to cancers of specific organs, and three forms of genomic instability have been identified in the gut. Chronic inflammation is implicated in the pathogenesis of most GI cancers, but it is not clear whether this is quantitatively sufficient to cause cancer by itself (2, 8, 10, 12).

Several genes are involved in the carcinogenesis of gastric cancer linked to cancers of specific genes. Three different types of genomic instability associated with gastric cancer have been identified in the gut. Chronic inflammation is implicated to pathogenesis of gastric cancer (15, 16). Gastric cancer is epidemiologically linked to chronic *H.pylori* infection, which is also responsible for chronic inflammation. Involvement of K-RAS mutation, loss of p53, inactivations of epigenetic and Epidermal Growth Factor Receptors (EGFR) are evidently found to be involved in the development of gastric cancer. However, there has not been any conceptual model involved in the sequential development of this disease as proposed for other gastro intestinal cancer. Interestingly, germline mutations in the gene of E-cadherin (an intracellular adhesion protein) are found in unique and rare form of familial diffuse gastric cancer (15). CDH1 and HER2 expression are also associated with hereditary diffuse gastric cancer (12).

Nearly 1-3 % of gastric cancer arise from inherited genetic susceptibility such as hereditary diffuse gastric carcinoma (HDGC), familial adenomatous polyposis etc. HDGC is an autosomal disorder with high penetrance. 30% of HDGC individuals shows germline mutations in tumour suppressor gene E-cadherin or CDH1(CDH1 is a classical member of the cadherin superfamily). In HDGC inactivation of the second allele of E-cadherin due to methylation, loss of heterozygosity or mutation triggers the development of gastric cancer. It is diffucult to diagnose HDGC at an early stage both

histologically and endoscopically mainly due to high penetrance of CDH1 mutation with the carrier of this gene which, confers for about 80% of the life time risk of gastric cancer. Importantly, according to the international gastric cancer consortium CDHI molecular testing is the recommended way for medical testing of gastric cancer (3, 10, 12).

HER2 (Human Epidermal Growth Factor receptor 2) gene amplification hasbeen identified in gastric cancer. HER2 is a 185kDa transmembrane tyrosine kinase receptor protein that regulates signal transduction in cell proliferation, survival etc. HER2 is a protooncogene of the EGFR and is located on the chromosome region of 17q21. Interestingly, HER2 overexpression is mainly observed in intestinal tye of gastric cancer or in cancers located at the proximal end of the stomach(2). Intestinal type of gastric cancer usually predominates in the patients with hereditary nonpolyposis colon cancer (HNPCC). HNPCC is due to disordered DNA repair caused due to mutations in the DNA mismatch repair genes like MSH2 (Mut2 protein homolog 2, is a tumour suppressor gene located in chromosome 2) or MLH1(MutL homolog 1 is located in chromosome 3 and is usually associated with nonpolyposis colon cancer). Gastric cancer is a common colonic manifestation of HNPCC as, patients with HNPCC show 12 to 13 fold increase in incidence in gastric cancer as cmpared to the genral population. Other inherited diseases that are closely associated with increased incidence of gastric cancer are Li Fraumeni syndrome (this syndrome is mainly caused due to germline mutation in TP53) etc.Familial adenomatouspolyposis, an autosomal dominant condition which is associated with APC mutation which often progresses to an overly malignant phenotype. Several cases of gastric cancer are reported in patients with APC phenotype (1, 7, 12).

Numerous chromosomal abberations have been reported in gastric cancer depending on the histological type. These aberrations are dependent on the overall survival and other clinicopathological parameters. Fusion proteins are common in malignancies. In gastric cancer a similar fusion protein involving the ROS1 gene (ROS1 gene encodes for transmembrane receptor tyrosine kinase). This fusion protein occurs as a result of genetic rearrangement and the occurence of such fusion proteins is quite less in case of gastric cancer. Occurance of microsatellite instability occurs in more than 50% of gastric cancer cases. Microsatellite instability results from impairment of DNA mismatch repair. Gastric cancer can be categorised depending on the status of microsatellite instability, gastric cancer patients with high

percentage of microsatellite instability has high overall survival compared to the stable or low microsatellite instability (1, 6, 12, 17).

Helicobacter pylori infection is pandemic all over the globe and has been associated with diverse clinical outcome. *H.pylori* infection is known to be associated with gastric cancer. The overall clinical outcome of gastric cancer largely depends on the overall distribution and severity of the infection induced by *H. Pylori*.

The human genome epidemiology systemetic review (HuGE) showed a close association between the severity of gastric cancer and inflammation related genes. For example the carriage of IL1RN*2 was associated with increased risk of gastric cancer whereas IL1B-31C was associated with reduced risk of gastric cancer. Mucin 1 (muc1) is an oncoprotein and a member of the mucin family (The mucins are a large family of heavily glycosylated proteins). MUC1 has been found to be relevant to the carcinogenesis of gastric cancer. Investigations revealed aberrant expression of MUC1 in gastric cancer tissues. MUC1, which is expressed on the surface of gastric epithelial cells helps in interaction with *H.pylori* during gastric cancer progression(3, 8, 12).

1.1.5 Current treatment for gastric cancer

Despite of major advances in understanding of gastric cancer biology the median survival of gastric cancer patients is less than 12 months, therefore development of personalised treatment strategies still remains the main challenge. First line chemotherapy is the standard treatment option for patients with advanced stages of gastric cancer with a good overall clinical outcome (4). In contrary, the overall performance outcome of combinational therapy in case of gastric cancer is muchless in comparisontosingleagent therapy. A meta-analysis in 2010 showed that combinational therapy showed a modest survival benefit of 1.5 months to that of single agent therapy. The standard chemotherapeutic regime for combinational therapy in gastric cancer patients usually involves older regimes like 5-Flurouracil/ anthracyclines in combinationwithPlatin, Irinotecanor Fluoropyrimidines like Capecitabine, S-1. 5-FU still remains the main backbone of combinational treatment in advanced gastric cancer. In Japan other than 5-FU S-1 is a widely accepted treatment option for patients with advanced gastric cancer for both single or combinational treatment (2,3,10). Overall patientsreceiving S-1 treatmentexperienced less side effects than 5FU treatment like grades of ¾ neutropenia was 32.3% for S-1

to that of 5FU with 63.3%, complicated neutropenia 5.0% versus 14.4% and somatits 1.3% versus 13.6%. Alternative treatment options as a replacementofCisplatinareof immense interest in gastric cancer cure at present. This is mainly because Cisplatin free regimes represent a more convenient therapeutic option and reduces the chances of hyperhydration or renal toxicity associated with cisplatin treatment. The use of oxaliplatin as an alternative option to Cisplatin is controversial. Al-Betran etal.,2008(14) conducted a study with 5-flurouracil (5FU) or leucovorin either in combination with oxaliplatin or cisplatin. This trial showed better tolerability of oxaliplatin in comparison to cisplatin. Whereas, in landmark REAL-2 trial revealed that oxaliplatin was not a superior option for cisplatin with epirubicin and either 5-FU or capecitabine treatment, as oxaliplatin treatment was found to be more toxic in comparison to these two standard chemotherapeutic agents. Irinotecan 5FU combinational treatment allthough, didnot show any overall beneficial time to progression outcome but the drug combination was well tolerated in patients with advanced gastric cancer (2, 3, 10, 17, 18).

The main aim of second or later lines of therapies is to improve the overall clinical outcome with little or no toxicity. In a Korean phase III trial the beneficial outcome of second or third line chemotherapies in advanced gastric cancer was confirmed. In this trial the patients were treated in a 2:1 ratio with chemotherapy (docetaxel or irinotecan) or BSC. The overall median survival improved significantly in patients withchemotherpy in comparisonto BSC. The efficacy of bothDocetaxelandIrinotecan was comparable (1, 2). Targeted therapies are often an effective beneficial option for cancer treatment. Allthough, as mentioned before HER2 expression is a characteristic signature of gastric cancer but currently the prognostic value of HER2 in advanced gastric cancer is controversial. Trastuzumab the standard chemotherapy for breast cancer is known to be an effective drug against HER2 as it interferes with HER2 expression. In a prospective randomised phase III trial showed Trastuzumab in combinatin with other chemotherapy in treatmentofgastriccancer.Trastuzumab in combination with cisplatin or S-1 hasshowninterestingoutcome. Therefore, the status of HER2 targeted therapy using Trastuzumab as a treatment option for advanced gastric cancer needs further investigations (17, 18). Lapatinib a tyrosinekinase inhibitor that blockes irreversibly to HER2 is an apporved second line treatment option for breast cancer. Randomised trials with Lapatinib in combinaion with Oxaliplatin etc didnot showanyadvantage in overall survival in gastric cancer

patients.For targeted therapy inhibition of Epidermal growth factor receptor 1 (EGFR1) was an alternative approach as 50% of gastric cancer tissues showed an overexpression of EGFR1. Clinical trials with Panitumumab (a human monoclonal antibody targeted against EGFR1) showedsignificantlyworseoverallsurvival in gastric cancer patients as a result the study needed to be terminated. Moreover, predictive biomarkers which could contribute to the efficacy of panitumumab could not be identified in gastric cancer (19). Anti angiogenic compounds like Paclitaxel, Ramucirumab showed significantly improved overall survival in gastric cancer patients. These data definately supports further development of anti-angiogenic agents in gastric cancer treatment (1, 2, 7, 16, 17, 18, 19).

1.2 Notch pathway

Notch signaling has been studied for centuries but recent development in genome wide or proteome wide investigations especially in model organisms has given a new surge and provided deeper insights into the Notch pathway. The basic characterisation of Notch signaling started with the discovery of Notch gene in *Drosophilla melanogaster*. Both vertebrate and invertebrate studies show that characterization ofNotchpathwayispleiotropic as it plays a fundamental role in cell fate determination and in all aspects of animal development. Importantly, Notch pathway has also been found to be involved in the development and progression of various cancers like pancreatic cancer etc (19, 20).

1.2.1 Notch signaling overview

Notch signaling is a critical pathway in metazoans that regulates important cellular activities like cell proliferation, differentiation and cell death. Notch is a cell surface receptor that sends short range signals via interactions with transmembrane ligands such as Delta commonly known as delta-like in human and serate comonly termed as Jagged in human present in neighbouring cells.This ligand binding leads to the cleavage and release of Notch intracellular domain (NICD). Translocation of NICD to the nucleus leads to the regulation of transcriptional complexes containing the DNA binding protein CSL (CBF1\RBPjK\Su(H)\Lag1), CSL is a DNA binding adaptor. CSL interacts with many proteins to form a repressor complex to preserve the closed chromatin confromation or activating complexes containng NICD. Followed by the synthesis, the Notch receptors are cleaved at site (S1) by protein convertases during

exocytosis. This in turn helps in the regulation of trafficking and signaling activity of Notch. While the passage through the Golgi different notch receptors get glycosylated by glycosyltransferses such as fringe. This in turn now determines the fate of response to various subfamilies of ligands. This along with other post translational modifications tune up the Notch activity in a context specific manner. Followed by ligand binding the activation of signal transduction occurs on endocytosis of ligand receptor complexes. Leading to the unfolding of the justamembrane negative control region also known as NRR (NRR is specific to Notch proteins). Unfolding of NRR results in the access of ADAM10 (a protease also known as KUZ). KUZ removes the NICD by cleavage at a specific site 2 or S2. Y-secretase (y secretase is a multi subunit protease complex that cleaves single pass transmembrane proteins within the transmembrane complex) then cleaves the transmembrane domain within Notch at site 3 (S3) resulting in the release of various forms of NICD. Interestingly, this productive interaction between the notch receptors and the ligands occur only when present in neighbouring cells i.e trans whereas when these receptors,ligands are present on the same cell i.e cis the interaction is inhibited. In canonical Notch signaling or also known as CSL mediated signaling NICD translocates to the nucleus and recruits adaptor proteins like Mastermind like (MAML). MAML inturn recruits histone acetyl transferase HAT p300. This activation leads to the transcription of Notch target genes such as the hairy and enhancer of split (HES) and Hes-related (HESR\HEY) family of basichelix-loop helix (bHLH).Thus everycleavednotch subunit reasembles into a single molecule which determines the NICD content present in the nucleus. During this transcriptional activation NICD is phosphorylated at its PEST domain by kinases like cyclin dependent kinase 8 (CDK8) followed by proteosome degradation by E3 ubiquitin ligases like Sel10 (also known as Fbw7) (Figure 3). This limits the half-life of Notch canonical signaling and resets the cell for next pulse of signaling (20, 21, 22, 23).

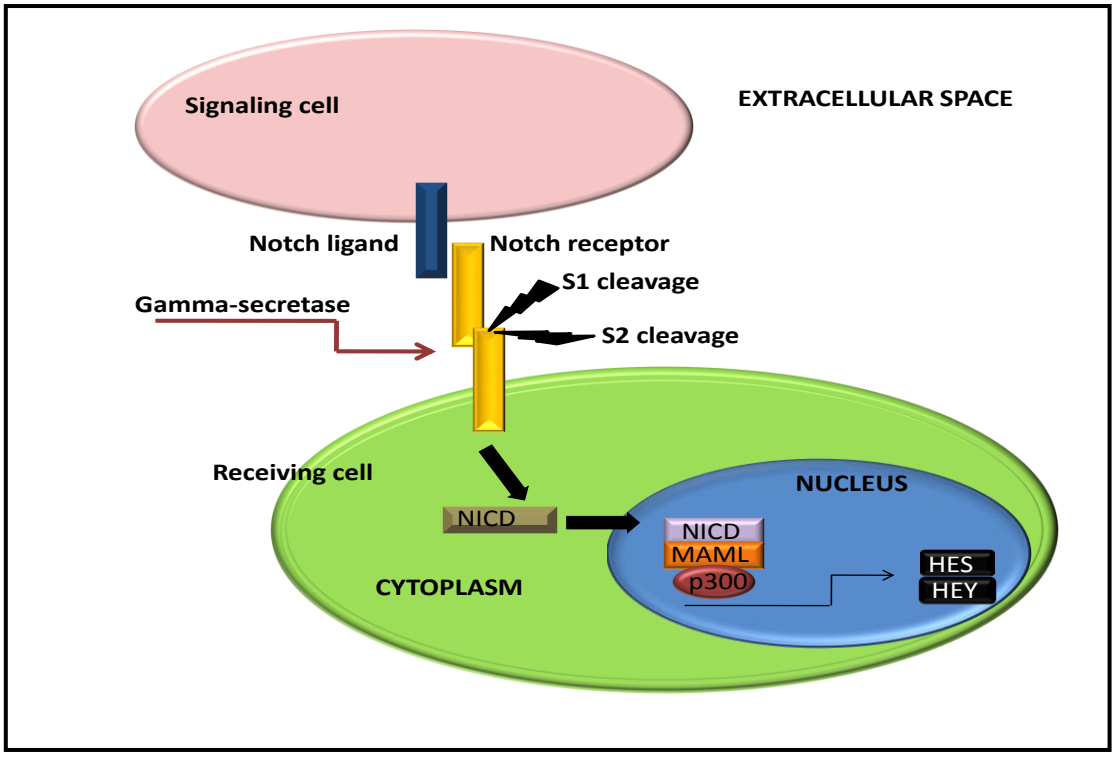


Figure 3: Schematic representation of Notch signaling. Notch ligand and receptors are expressed on cell surface. On interaction or binding of the Notch ligand to its receptor on an adjacent cell leads to a series of proteolytic cleavages referred as S1 and S2 cleavage by gamma secretase enzyme. This results in the release of Notch intracellular domain (NICD) that subsequently translocates to the nucleus. On nuclear translocation of NICD, Notch signaling is activated NICD binds to CBF1 followed by recruitment of nuclear protein MAML, MAML recruits histone acetyl transfease protein P300 , this results in the transcriptional activation of various Notch target genes belonging to HES and HEY families (Adapted from 28).

Several studies have also revealed the existence of non-canonical Notch signaling pathway. Other signaling pathways can also be activated by cleaved NICD but independent of CSL. Interestingly, other signaling pathways like JAK/STAT, RTK, MAPK, FGF, Wnt etc can activate the non-canonical Notch signaling via activation of common Notch downstreams targets like Hes family oftranscriptionfactors etc. It is evident from some studies that such interactions of activation of non-canoncal Notch signaling pathway play role in both development and disease (20,21, 22, 23, 24).

1.2.2 Notch in development and disease

Notch signaling pathway is one of the basic signaling pathways involved in development for both cell types and organogenesis. The role of Notch signaling pathway in drosophila was first studied due to the recognition of a dominant Notch winged phenotype in drosophilas. The importance of Notch signaling in human development and disease was first reported when mutations in Notch1 was identified to be associated with occurance of T-cell acute lymphoblastic leukeamia (T-ALL). Since then development of other developmental disoders associated with Notch signaling has been identified and different animal models has been developed to understand how different components of Notch signaling contributes to the development of various organs associated with these diseases (22, 25, 26, 31, 33).

Notch3 mutations resulting from the loss or gain of cysteine residues in the epidermal growth factor like repeats was found to cause CADASIL (a disorder characterised by stroke and dementia).Pathological studies has shown accumulation of Notch 3 in braintissuesofthesepatients. Unfortunatley, the precise mechanism for this disorder is not known (22, 25, 27).

Alagille syndrome is a multi system, genetic disorder that mainly effects the liver, heart and kidney. JAG1 mutations were identified in this disease which shows the pleiotropic function of Notch signaling in mammals.

The ALGS liver disease requires most consistent medical treatment and is associated with various liver phenotypes. JAG1 or Notch2 mutations were found in the liver phenotypes seen in ALGS resulting in lethality/paucity in bile duct formation. This is mainly because Notch signaling plays an important role in bile duct formation, therefore mutations in Notch components leads to lethality in bile duct formation resulting in ALGS phenotype. ALGS phenotype also causes other anomalies like renal, vascular, cardiac etc (22, 25, 26, 27).

Medulloblastoma is also known as malignant brain tumour in childhood. Several groups have reported the potential role of Notch signaling in medulloblastoma; studies showed the increased mRNA expression of Notch2 and its target gene Hes1. This coexpression was found to be associated with overall poor patient survival. Thus, Notch signaling can be a therapeutic target in treating medulloblastoma (22).

The role of Notch in differentiation, morphogenesis and development of central nervous system has been recently identified. Studies show that inactivation of Notch signaling elements leads to altered neurogenesis. In case of CNS altered neurogenesis mainly occurs due to low expression of notch downstreams like Hes1and Hes5 this results in the inability to generate NICD (22, 25). Notch signaling is also known to play a pivotal role in endocrine development i.e pancreas, gut and bone. Specific Notch pathway downstream elements are expressed during pancreas development. Thus,suggesting the role of Notch signaling in pancreatic development. Loss of function of a series of Notch target genes like Delta1, and Hes1 results in the accelerated or upregulated differentiation of pancreatic endocrine cells. Notch target genes have been found to be involved in the early intestinal cell fate decision. Different notch target elements are expressed in murine crypts specifically Hes1 is expressed in crypts. Math1 mutant mice (a direct target gene of Hes1) died neonatally. Thus it shows that Hes1 and Math1 genes are important in determining or distorting the fate of differentiating cells (22, 25, 27).

Intestinal epithelial cells shows an overexpression of NICD. Microscopic examination of the early developmental stages of the intestine revealed that NICD expression effects the overall architecture of the villi and also decides the fate of differentiated secretory cell lineage. Importantly, transcriptional analysis showed a direct connection between NICD overexpression and elevated levels of Hes1 transcription in the intestinal epithelium of the Hes1 transgenic mice. Genetic data thus, clearly shows that Notch mediated Hes1 expression determines the binary fate of adsorptive and secretory cells. Hes1 deficient mice didnot show any change in the proliferation of the intestinal precursor pool (22, 25, 26, 28).

Osteoclastogenesis is a process of development of osteoclasts (osteoclast is a bone cell type that breaks down bone tissue). Osteoblastogenesis is a process of development of osteoblats (osteoblasts are cells with single nuclei that synthesize bones). Evidences show that Notch pathway regulates both osteoclastogenesis and osteoblastogenes is during bone development. Notch activation downregulates the expression of c-Fms (c-Fms is a receptor for macrophage colony stimulation factor) in osteoclast precursor cells and also reduces the expression of osteoprotegerin ,also known as osteoclastogenesis inhibitory factor (OCIF) in stromal cells. This results in the downregulation of osteoclastogenesis. Moreover, continuous NICD expression leads to the inhibition of osteoblast differentiation in osteoblast precursors.

Concomitant activation of Delta1 and Jagged1 has also been observed *invivo* during bone regeneration. All these observations together suggests a potential role of Notch pathways during bone regeneration and also in osteoporosis (22, 23, 25, 26).

1.2.3 Notch signaling in cancer

After the discovery of involvement of Notch signaling in T-ALL, the role of Notch signaling has also been implicated in various solid tumours like meduloblastoma, breast cancer, melanoma, non-small cell lung carcinoma etc. The oncogenic potential of Notch was first observed in Mouse Mammary Tumour virus (MMTV) driven breast cancer. Characterisation of MMTV driven breast cancer led to the observation of truncated levels of Notch 4. Importantly, in human breast cancer overexpression of Notch or Jag1 was observed and this overexpression directly correlated with poor prognostic outcome. Moreover, few activating mutations of notch has also been observed in the solid tumours of Non small cell lung carcinoma (NSCLC). Recent studies have also revealed Notch1 mutations in Chronic lymphocytic lymphoma (CLL). All though Notch1 mutations were not observed to be pathognomonic in development of CLL but led to poor patient survival. Therefore, targeting notch signaling could be of therapeutic interest in this distinct clinical subtype. (23, 29, 31, 32, 33, 36)

In skin Notch activation induces differentiation and cell cycle arrest. Loss of function of Notch1 resulted in basal cell carcinoma that appeared specifically in older mice. Thus Notch acts as a tumour suppressor in skin but the probable mechanistic approach still needs to be determined. Hepatocellular carcinoma (HCC) is one of the most devastating cancers worldwide. HCC mice model showed elevated levels of Notch pathway thus, activation of Notch is associated with HCC progression. Lobry et al., 2011 (29) showed that Notch loss of function or compound deletion of Notch1 and Notch2 resulted in a myeloproliferative syndrome. This syndrome showed common features with chronic myelomonocytic leukemia (CMML). Stransky et al.,2011 (27) and Agrawal et al.,2011(33) identified 28 different Notch1 mutations in 17.5 % of head and neck squamous cell carcinoma (HNSCC) and also Notch2, Notch3 mutations in 11% of the patients. Mainly loss of function mutation of the notch receptors was observed in HNSCC patients. In contrast to the oncogenic potential of Notch B cell malignancies reported that Notch suppressed growth and induced apoptosis in these cells. Thus providing additional information that Notch

could also act as a tumour suppressor in hematopoietic cells. In neuroblastoma similar tumour suppressor function of Notch was observed. Thus, these studies together suggest that Notch can also act as a tumour suppressor in some dieased models (23, 29, 31, 32).

1.2.4 Notch signaling in gastric cancer

In normal gastric mucosa Notch signaling is involved in the process of differentiation of the gastric epithelium into foveolar glands. Studies have shown expression of Notch1, Notch3, Jagged1, Jagged2 and Hes1 in the isthmus region of the normal mucosa where usually putative gastric cancer stem cells are found. Interestingly, Notch signaling is involved not only in the differentiation of normal gastric mucosa but also it is found to be involved in the differentiation of gastric cancer cells. Human gastric cancer tissues have shown expression of Notch receptors like Notch1, Notch2, Notch3 and also of ligands like Jagged 1 etc. Expression of Notch1 for instance is observed in both the premalignant and cancer tissues, specially in well differentiated intestinal gastric cancer. Piazzi et el.,2011 (30) showed that activated Notch1 has the potential to form solid tumours in human gastric cancer xenografts. This group also showed that increased expression of Delta-like 1, (DDL1, a ligand of Notch1) was associated with the increased activation of Notch1 and increased expression of NICD domain, Hes1 in gastric cancer cells. Importantly, in gastric cancer patients a strong correlation between DDL1 and Hes1 expression was observed. This correlation was found to be associated with overall patient survival. Patients with high expression levels of Notch3 and Jagged 2 show better clinical outcome to treatment as compared with patients with low level of expression. Therefore, Notch receptors and ligands could be a potential therapeutic targets for gastric cancer treatment. Studies also revealed that specially increased expression of Jagged 1 was clearly associated with aggressiveness of the cancer and resulted in overall poor patients survival. Thus, Jagged1 can act as an effective prognostic marker for gastric cancer. Inspite of knowing the contribution of Notch signaling receptors and ligands in gastric cancer progression, the exact mechanistic approach of Notch signaling cascade in gastric cancer still remains unknown. Some studies show that Notch1 activation in gastric cancer progression could be partially attributed to cycloxygenase2 (COX2). On treatment of gastric cancer cells with COX2 inhibitor showed impairment of migration, invasion and colony formation. Therefore, these studies clearly show that Notch signaling is involved in the pathogenesis of gastric

cancer but the level of expression of Notch ligands, receptors is cell type or circumstance specific. On a whole a better understanding of Notch mediated malignant transformation in gastric cancer could be useful in developing novel anti-cancer strategies for gastric cancer treatment (28, 31, 32 ,34).

1.2.5 Notch inhibition strategies

Inhibition of Notch signaling specially in cancer is complicated due to the fact that pathway members donot have enzymatic activity themselves and mostly it is easier to develop small molecule enzyme inhibitors. In recent times inhibition of Notch signaling can be achieved by several different options as follows (Figure 4) (17):

1) Small molecule inhibitors

Canonical signaling of Notch pathway requires two series of enzymatic cleavage of activation first by alpha secretase complex and then by gamma secretase complex. This enzymatic activity can be inhibited by using small molecule inhibitors, Gamma secretase inhibitors (GSIs) represent the pioneering class of Notch inhibitors. GSIs were primarily developed for Alzheimer‘s disease but were later adapted for cancer therapy. Importantly, GSIs have other targets than Notch receptors and ligands like CD44, E-cadherin, N-cadherin, Nectin 1 alpha etc (35, 36).

2) Other potential approaches to small molecule inhibitors

As an alternative to GSIs Alpha secretase inhibitors also known as ASIs has been developed. There function is not only attributed to the inhibition of notch signaling but ASIs are also knwon to be involved in impairment of ADAM (A Disintegrin And Metalloprotease). ASIs are known to be superior to GSIs:ASI does notneedtoenter the cell to act unlike GSI. ASI as a potential Notch inhibitor in cancer is still undertesting.

It is possible to develop different small molecule inhibitors for Notch which can act in different fashions, as inhibiton of enzymatic activity is one of the most feasible way to achieve blockage of a specific protein or pathway. Recent studies show that there are likely to be other points of Notch pathway amendable to blockage. This alternative approach relies on the fact that gamma secretase cleavage of Notch is dependent not on the cell membrane but also in the acidic endosomes. The Na+/H+ antiporter Monensin emerged as a potential acidification inhibitor for Notch. The

supremacy of such inhibitors in comparison to GSIs in terms of specficity and effectivity still remains to be determined.

A number of protein protein interactions involved in the Notch pathway like Notch-Notch ligand etc could be potential targets to achieve inhibition of Notch pathway. Such a small molecule inhibitor involved in blocking of protein protein interaction is nutlin (nutlin interferes with p53\MDM2 interaction) (12, 21, 36).

3) Antibody inhibitors

Antibody inhibitors are a prominent means to interfere or block protein-protein interaction. One advantage of antibody inhibitors is their specificty to target each Notch family members or ligands involved in Notch activity. For some cancers local delivery is sufficinet as a curative option but for metastatic malignancies it is essential to have a systematic distribution. Therefore, antibody inhibitors of Notch are an effective approach for haematological malignancies or angiogenic uses as it requires effective distribution of the drug.

Inhibition of Notch signaling via blockage of Delta like 4 ligand leads effective anti-angiogenic strategies. Therefore blocking of Delta like 4 ligand with specific antibodies led to the impairment of tumour angiogenesis resulting in dysfunctional vasculature and tumour regression. Studies show that development of specific antibodies to different Notch family members can be beneficial in inhibiting cancer growth. For instance antibodies to Notch 3 that can block the Notch signaling cascade by blocking the target site for alpha secretase binding. This is achieved either by exposing the target site or by reinforcing its blockage. Usage of such Notch specific antibodies may be beneficial as a new modality to treat cancer with increased effectiveness and less toxicity (36, 37).

4)Novel methods for Notch inhibition

Notch inhibition can be achieved by the above mentioned strategies but recently the development of alternative new strategies are also emerging as an effective approach. These new approaches include usage of stapled peptide which blocks the interaction between Mastermind-like with NICD. This strategy of Notch inhibition was used by Verdine and Korsmeyer labs at Harvard by the development of a bcl2 inhibitor. Recent reports have described the usage of a stapled peptide derived from

the alpha-helix of MAML protein termed as SAHM1. SAHM1 has the potential to block Notch canonical signaling which could be an effective therapeutic option for treating Notch dependent hematopoietic cancers such T-cell actue lymphoblastic leukemia etc (36).

Geneticstrategiestoinhibit Notch signaling has also been developed, this usually involves delivery of a pseudo gene which encodes for a Notch inhibitory protein or peptide. Genetic inhibition startegies have limited application specially for haematological malignancies or specialised tumours.

RNA interference is another strategy used recently but with more potential as a therapeutic startegy. Delivery of Notch inhibiting genes isthe main challenge therefore using RNA intereference which mainly involvesdeliveryof small oligonucleotides rather than the whole gene is comparitively less challenging. RNA interference usually involves usage of micro RNAs (miRNAs) or small-interfering RNAs (siRNA). For example miR-326 is used to block Notch1 and Notch2 activity to inhibit Notch dependent cancer development. MicroRNAs offer the potential to suppress the activity of more than one target gene but the suppression is not as effective as siRNA mediated silencing of target genes for cancer cure. Therefore, whether usage of siRNA or miRNA could be a more effective approach to treat cancer still remains an open question (36, 37).

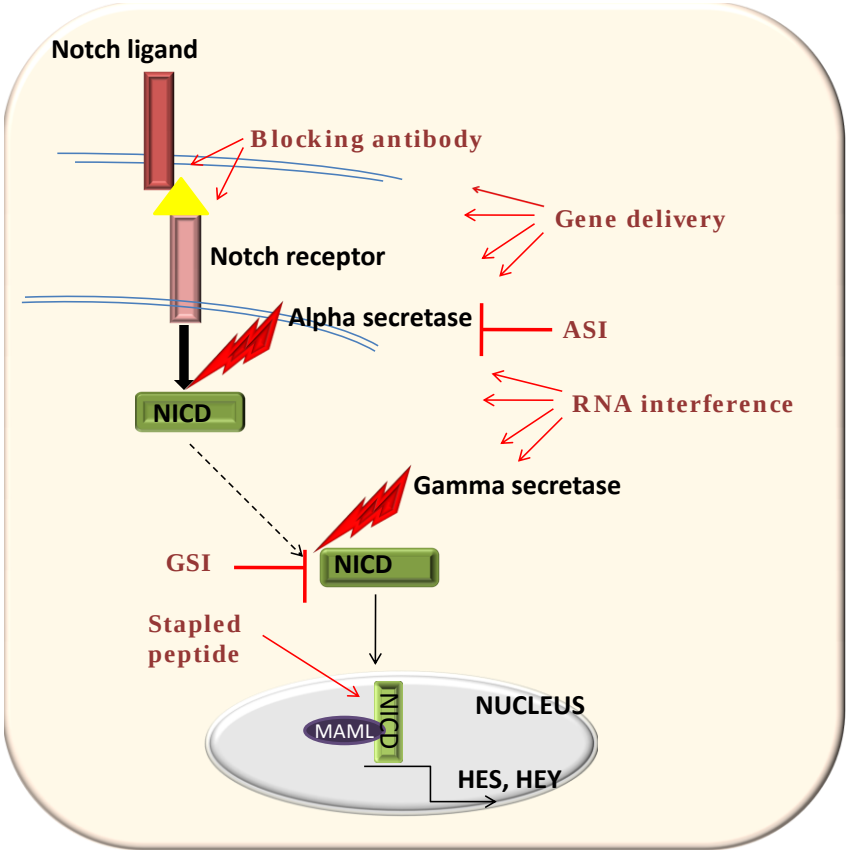


Figure 4: Notch inhibition strategies.

Different approaches of Notch inhibition in cancer therapy. ▲ – showing blocking antibodies in order to inhibit Notch receptor ligand interaction. Alpha secretase and gamma secretase inhibitors to inhibit alpha and gamma secretase enyzmatic activity for release of Notch intracellular domain and its translocation to the nucleus. Gene delivery ,RNA interference and usage of stapled peptides to suppress notch target genes like Hes, Hey etc. Abbreviations: NPR- negative regulatory region; NICD- Notch intracellular domain; ASI- alpha-secretase inhibitor; GSI- gamma-secretase inhibitor; MAML - Mastermind-like; HAT- Histone acetyl-transferase (Adapted from 36).

1.3 CD44 in cancer stem cells

A long standing goal has always been to understand which cancer or tumour cells need to be eliminated via treatment to achieve successful outcome. One framework that has received enough attention regarding this aspect is the “cancer stem cell hypothesis”. This hypothesis states that the cancer cells also have a sequential or hierarchial developmental structures which inculde some cells which can proliferate indefinately, these cells are charecterised as cancer stem cells. Therefore, targeting such cancer stem cell population could be of immense importance for cancer treatment. In order to target these cells, cell specific surface markers are required.

This concept led rise to cancer stem cell (CSCs) markers. Amongst several other markers CD44 is a widely used cell surface marker for cancer stem cells(38).

1.3.1 Cancer stem cells (CSCs)

A Tumour is not a mass of homogenous cells but also includes several different cell types like haematopeitic cells, enodthellial cells, stromal cells which can influence the function of the tumour as a whole. These heterogenous cells impact the tumour microenvironment like hypoxic environment etc which causes heterogenicity in tumour functions. Recently, studies are being conducted to reveal the underlying mechanism of tumour heterogencitiy and its impact on overall resistance to therapy, tumour progression or recurrence. According to the cancer stem cell theory within this heterogenous mass of tumour cells lies a subpopulation of self-renewing cancer cells known as the cancer stem cells (CSCs). Both experimental models and clinical studies show that CSCs are resistant to chemo or radio therapy (Figure 5). The above mentioned properties and transcriptional signatures are highly specific to CSCs and are also highly predictive in overall patient survival. Thus CSCs also have a huge clinical relevance. Recentfindings in Leukeamiaand solid tumours indicate that the gene signatures specific to cancer and normal stem cells are highly prognostic for a wide spectrum of patients. In 1961 Till and McCulloch performed an *in vivo* clonal assay where he showed that one single clone of haemopoeitic cells were capable of undergoing multilineage differentiation but still retained the property of self renewal. Self-renewal property is often termed as "stemness" ofthecell. Stemness is an important characteristic property of stem cells. Stemness or the property of self-renewal is a key biological process in which one stem cell divides into one or two daughter cells which retain the capacity of self renewal. Therefore, ensuring that the stem cells population is maintained intact (38, 40, 41).

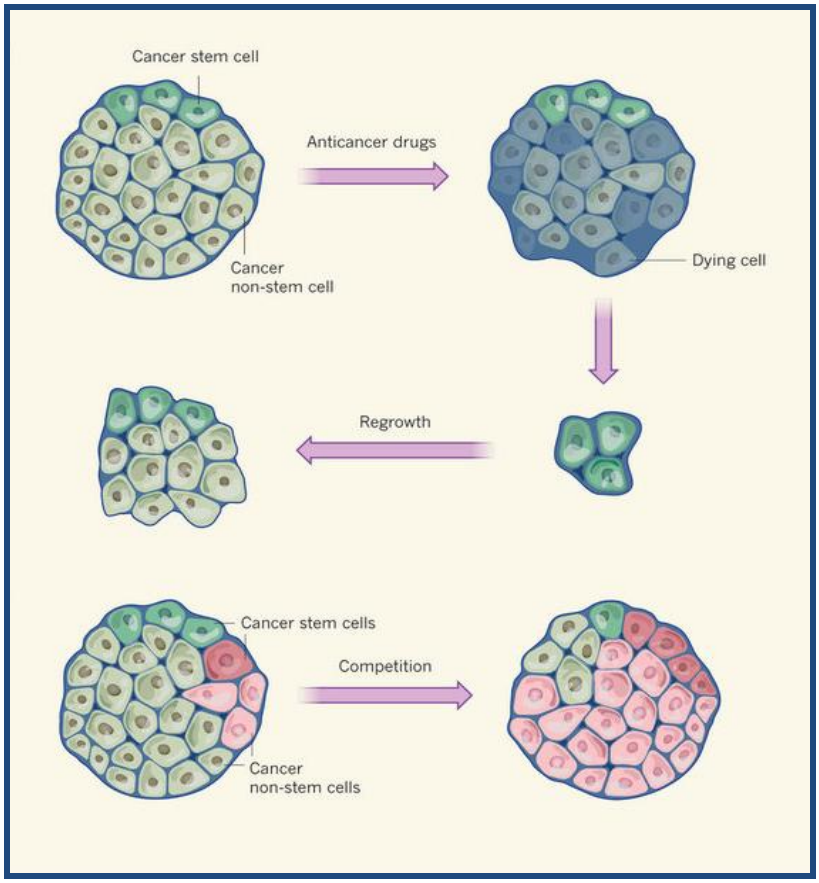


Figure 5: Stem cell in tumour.

Heterogeneous mass of tumour tissue containing CSCs. Anti cancer drugs can target and kill the normal tumour cells but not the resistant cancer stem cells. These cancer stem cells causes relapse of tumour growth (Adapted from 42).

Both CSCs and normal tissue stem cells both posses the property of self renewal but during carcinogenesis the self renewal property is typically deregulated. The basic defining feature of CSCs is they can be identified as a distinct population from the rest of the tumour possesing the potential of repopulation and self renew. CSCs on the basis of their self renewal property are also named as Tumour intiating cells (TIC) .TICs can be identified for their specific characteristics: Firstly, the potential of TICs to form xenograft tumours secondly, to self renew and finally, to produce daughter cells with the ability to proliferate. There are several evidences which show a strong linkage to stemness and therapy outcome as CSCs not only show resistance to cancer therapies but also invasion of cell death and dormancy as observed in colon cancer, breast cancer etc. Recently, it has been observed that in multiple myeloma CSCs are more resistant to a proteasome inhibitior used for treating the malignancy as comapred to the other bulk tumour cells. Emerging evidence also suggests a

strong linkage to stemness and prognosis or therapy failure. This shows targeting CSC population can be of interest to eradicate the resistant self renewing population of CSCs present within the tumour and also to reduce the chances of relapse.In order to achieve specific targeting of CSCs identification of tumour specific CSC markers are of utmost importance. Recent studies have identified several cell surface marker for CSC like CD24, CD44, CD133 etc. Therefore, understanding the stemness property present within the tumour we are able to gain insight into the most important cell population present within the tumour capable of maintaining sequential rounds of tumour growth (38, 39, 40).

1.3.2 CD44

CD44 is a transmembrane glycoprotein that is known to have several functions in cell division, migration, adhesion, angiogenesis, presentaion of cytokines etc. CD44 binds to its primary ligand Hyaluronic acid (HA). This binding leads to activation of cell signaling and also other important regulatory process within a cell. All of these process regulated by CD44 are important for normal cells but are also pivotal for cancer cells. CD44 being a cell adhesion molecule helps in cell-cell signal transduction. CD44 also regulates the signal transduction of common cell regulatory pathways like human epidermal growth factor (HER2) and MET receptor tyrosine kinase etc and leads to the activation of the downstream signaling cascade associated with important cell regulator processes. It has a molecular weight of 85-200 kDa. Interestingly, binding of CD44 also results in stem cell homing. CSCs generate HA (the primary ligand of CD44 as mentioned above) in order to attract macrophages to the CSC niches. These CSCs in turn produce oncogenic growth factors which help to maintain the proliferative phase of the tumours. Moreover, interaction of CD44 with HA is also crucial for cellular invasion. *In vitro* studies of breast cancer cells have shown that metastasis can only be prohibited via preincubating the invasion chambers with anti CD44 oligos. This shows that CD44 HA interaction is a pivotal process for invasion. However, how HA effects invasion still remains unclear.These evidences taken together shows that CD44 is expressed in CSCs and thus can be used as a marker to target CSCs. Experimental studies have also shown that targeting CD44 via usage of CD44 antibodies or CD44 soluble proteins have reduced the maliganant activities in several neoplasms (44, 45, 49).

CD44 has 20 different isoforms generated due to alternative splicing. These different CD44 isoforms participate in various cell regulatory processes. The most commonly known isoforms of CD44 comprises of 12 isoforms. These isoforms are denoted as variants therefore, symbolised as "V" and the nomencleture is CD44v# (Figure 6). These variants can be isolated from normal cells and can also be obeserved in pathological cells. Out of these 20 different isoform CD44H is the most standard and simplest isoform. CD44H is well distributed since, it is a haematopoeitic isoform as it is found in whiteblood cell, natural killer cells etc it is denoted with H. Importantly, CD44 isoforms are also expressed in certain cancer metastatic stem cells. CD44v6, one splice variant of CD44 is known to be expressed and also known to be involved in breast and colorectal cancer. On a whole thus it can be concluded that CD44 along with it ligands can modulate adhesiveness, motility etc and can also allow a tumour cell to proceed through all the steps of metastatic cascade (47, 49).

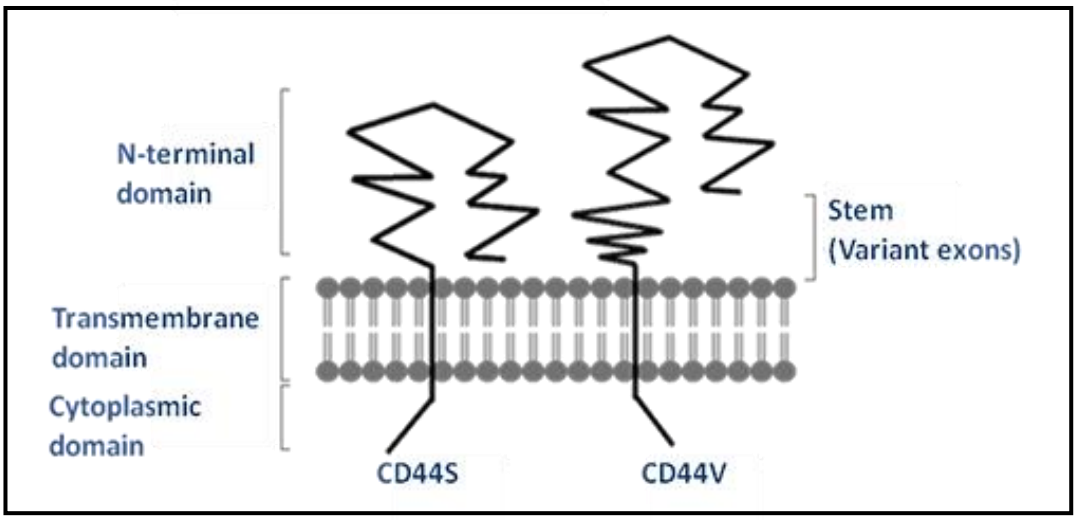


Figure 6: CD44 splice variants.

The basic outlook of CD44 splice variants (CD44 S and CD44 V). CD44S contains the basic domains (N-terminal domain, transmembrane domain and cytoplasmic domain).But CD44V has an additional longer stem containing variant exons (Adapted from 48).

1.3.3 CD44 in gastric cancer

Interest in gastric cancer stem cell has arisen in a broader context to the CSC hypothesis for tumour progression in cancer research. The new paradigm is

remarkable in the context that current anti-cancer therapies are more successful on the non CSC population of tumour cells compared to the CSCs. Thus identification and charecterisation of CSCs in cancer can be beneficial for therapeutic intervention. The *in vitro* studies by Takaishi and colleagues (50) as well as several other studies have identified CD44 as a cell surface marker for gastric CSCs. $CD44^+$ population of gastric cancer cells showed property of self-renewal which is consistent with stem cell phenotype. Moreover, $CD44^+$ cells showed resistance to both radio and chemo therapy thus reflecting them as an attractive target for gastric cancer treatment. Interestingly, among other CSC markers like CD133, CD24 etc majority of gastric cancer cells expressed more CD44 as compared to other markers being as high as 95% (50, 51, 52) (Figure 7).

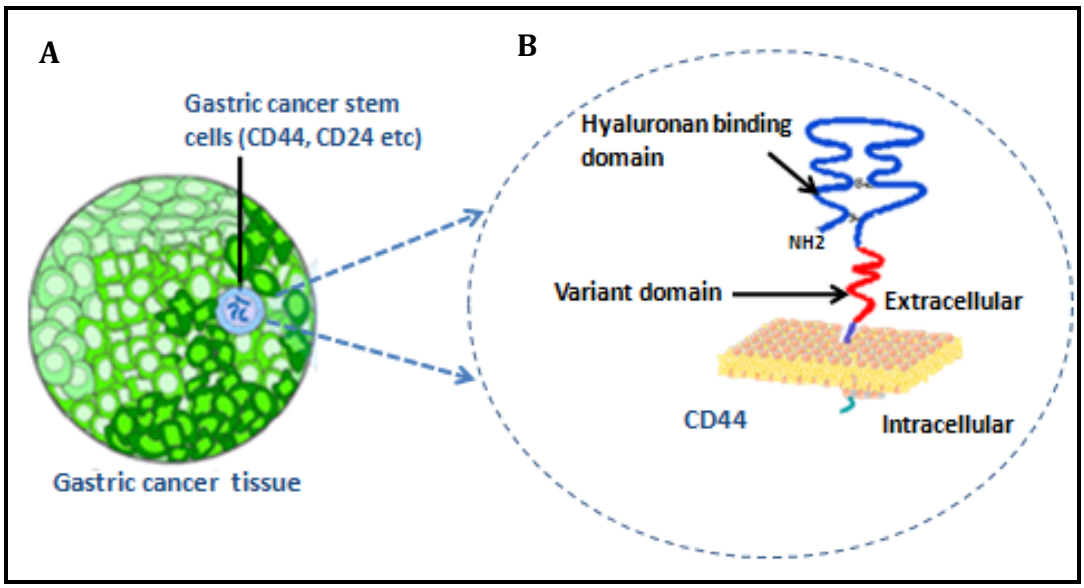


Figure 7: CD44 in gastric cancer.

A.Gastric cancer tumour mass containing gastric cancer stem cells like CD44, CD24.**B**. The detailed structure of CD44 containing the hyaluronan binding domain and variant domain (Adapted from 53).

Taken together the studies suggest that CD44 fraction contain gastric CSCs. As CD44 is also present on non-tumorogenic gastric cancer cells therefore, identification of additional markers might also be helpful for narrowing down and identifying gastric CSCs. CD44 and its variants have been identified in gastric cancer and these variants have shown a correlation with poor prognosis specifically in patients identified with intestinal type gastric adenocarcinoma. Lau and his colleagues (51)

identified CD44 splice variant containing variable exons 8,9 and 10 were significantly elevated in gastric tumour compared to normal gastric tissue. Morover, they show that amongst the CD44 expressing gastric cancer cells, CD44 variant 8 to 10 is the major variant found in gastric CSCs. CD448-10 is also found to play a functional role in gastric tumour initiation (45, 51, 54, 61).

1.3.4 CD44 inhibition

Understanding the role of CD44 in cancer progression led to the evolution of CD44 as an attractivetargetin the field of cancer therapies. Several reports shows that inhibition of CD44 led to beneficial results in cancer treatment. Therefore, various approaches to inhibit CD44 in cancer progression was developed as an alternative strategy to treat cancer.

Aptamers have been used as an alternative to antibody based therapies Lida J et al., 2014 (56) developed an DNA aptamer Apt#7 which binds to CD44 exon v10 and thus blocks the formation of CD44-EphA2 complex associated with breast cancer migration. Apt#7 aptamer inhibited migration of breast cancer cells at comparatively low dosages like 5 to 10nM. Apt#7 aptamer not only inhbited migration but also invasion and inhibited various tyrosine kinase pathways associated with breast cancer development (56, 57).

Studies showed a strong association in squamous cell carcinoma between CD44v6 and tumour progression. There, a humanised monoclonal antibody against CD44v6 named as Bivatuzumab was developed. Bivatuzumab was coupled with a cytotoxic drug mertansine in order to ensure effective treatment was used in phase I dose escalation studies in patients with head and neck squamoous cell carcinoma. This combinational treatment via inhibition of CD44 was found to be effective in 2 out 20 patients undergoing the clinical trial. A similar study using radiolabelled humanised antibody against CD44v6 was developed for treating patients with early stages of breast cancer. Unfortunately, during clinical trials this drug needed to be terminated due to accumulation of the drug in non-tumorous areas and also failed to inhibit CD44v6 expression in breast cancer.

A peptide against CD44v6 was developed with the potential to inhibit the association between CD44 MET and VEGFR2 (Vascular Endothelial Growth Factor Receptor 2) in endothelial cells. This v6 blocking peptide effectively inhibited angiogenesis and

metastasis in pancreatic cancer xenografts but this peptide has not been tested in clinical trials yet. The miRNA miR34a was recently identified as a regulator of CD44, miR34a expression resulted in degradation of CD44. The use of tmiR34a as a strategy to inhibit CD44 expression effectively inhibited tumour growth and metastasis in mice with prostrate cancer. Moreover, these miR34a treated prostrate cancer mice also showed overall increased survival (46). Natural compounds have also shown to be effective in inhibition of CD44.BXL0124 (a novel gemini vitamin Danalog) showed impairment of tumour growth and CD44 expression in human breast cancer both *invivo* and *in vitro*. It was observed that this natural compound effectively inhibited breast cancer growth via Vitamin D receptor and p53 dependent mechanisms (58, 59).

1.3.5 Targeting pathways in CSCs

Gaining insight about the mechanisms involved in the maintenance of self renewal potential is of importance for development of anti cancer drugs and targeting CSCs. Reports reveal that wnt, Notch and Hedgehog are the threemain pathways that might be involved in the recurrance and maintenance of CSCs. NF-κB, phosphatidylinositol 3-kinase (PI3K)/Akt, PTEN are also some of the common pathways which show aberrant activation in CSCs. It is believed that these pathways provides CSCs the potentail to initiate cancer and also promote recurrance after the surgical removal of the tumour (60).

Hedgehog pathway is one of the key pathways known to be involved in the maintenance of CSCs. Canonical hedgehog signaling involves binding of three main hedgehog ligands Sonic (SHH), Indian (IHH), and Desert (DHH) to patched receptors. The ligands are differently expressed depending upon the cell type. This binding leads to the activation of transcriptional effectors of GLI family (50, 58, 60).

Studies reveal the involvement of wnt signaling in the self renewal and maintenance of CSCs in several tissues like skin, intestine etc. Importantly, a subpopulation of tumour initiation cells associated with drug resistance showed activation of Wnt signaling. Inhibition of wnt signaling in these population led to the blockage of breast tumour growth in murine models. wnt signaling is characterised by the canonical wnt pathway or the beta-catenin dependent pathway. Wnt signaling usually involves Wnt ligands, Frizzled receptors and formation of a complex composed of APC, Axin1, Glycogen synthase kinase 3-β, and CK1 (casein kinase 1). Formation of this complex

leads to the stabilisation of beta-catenin which stimulates mediators controlling cell movement and behaviour (Figure 8). *In vitro* studies have showed that usage of beta-catenin nuclear transfer inhibitor not only decreased significantly the colony formation of lung carcinoma cells but also importantly, downregulated pluripotent stem cell signaling pathway. Studies have shown that usage of wnt inhibitors canpotentially eliminate the drug resistance associated with CSCs (58, 60, 63).

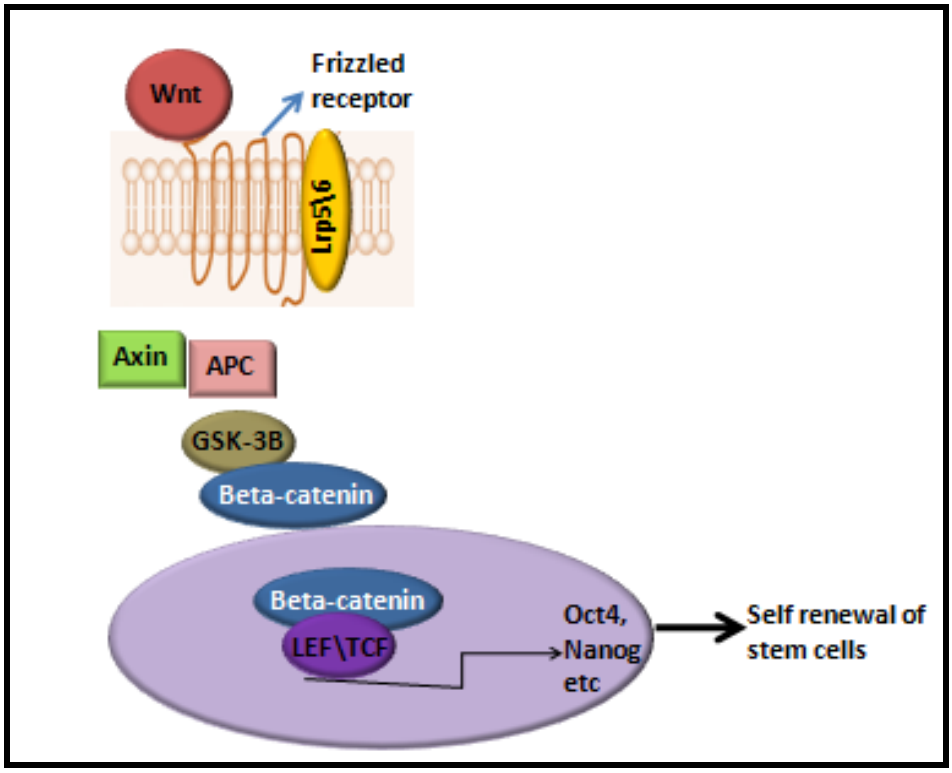


Figure 8: Schematic representation of the overview of wnt signaling in maintenance of cancer stem cell.

Wnt protein transduces extracellular message to intracellular signaling cascade by binding through frizzled receptors. β-catenin forms destruction complex (Axin1, 2/APC/CK1α and GSK-3β,). Phosphorylation of β-catenin within this complex leads to ubiquitin and proteosomal mediated degradation process. In the presence of wnt ligand wnt protein binds to frizzled receptors along with its LRP5/6 co-receptor complex leading to interruption of the destruction complex. The activated cytosolic accumulation of β-catenin, leads to translocation of beta-catenin to the nucleus. In normal stem cells, the Wnt pathway signaling causes activation of its target genes such as OCT4, Nanog and Sox-2 leading to maintenance of self-renewal property (Adapted from 59).

Interestingly, several reports show that amongst all of the above mentioned signaling cascades associated with CSC maintenance specifically Notch and wnt-beta-catenin pathways are found to be significantly active in gastric CSCs associated with gastric carcinogenesis, prognosis and chemotherapy resistance. Therefore targeting and impairment of these two pathways specifically could eradicate the targeted CSC population resulting in an overall effective clinical outcome (75, 83).

1.4 Gamma-secretase inhibitor (GSI)

Gamma-secretase is basically a large complex composed of two subunits: a catalytic subunit (consisting of presenilin-1 or presenilin-2) , accessory subunit (containing pen-2, Aph1, nicastrin) (63). The pivotal role of gamma secretase has been evaluated in the activation of Notch signaling, it hasbeen observed that point mutation in the cleavage site of Notch 1and also double knockout of presenilin-1or 2 led to embryonic lethality in mice. Therefore, as gamma secretase inhibitors (GSIs) are able to block Notch receptor activation, several GSIs havebeen tested for its anti-tumour effects (Table 1).

Gamma-secretase is known to have several other substrates other than Notch ligands like ErbB4, Sydecan (an extracellular matrix) and most importantly CD44 (35, 37, 41, 63).

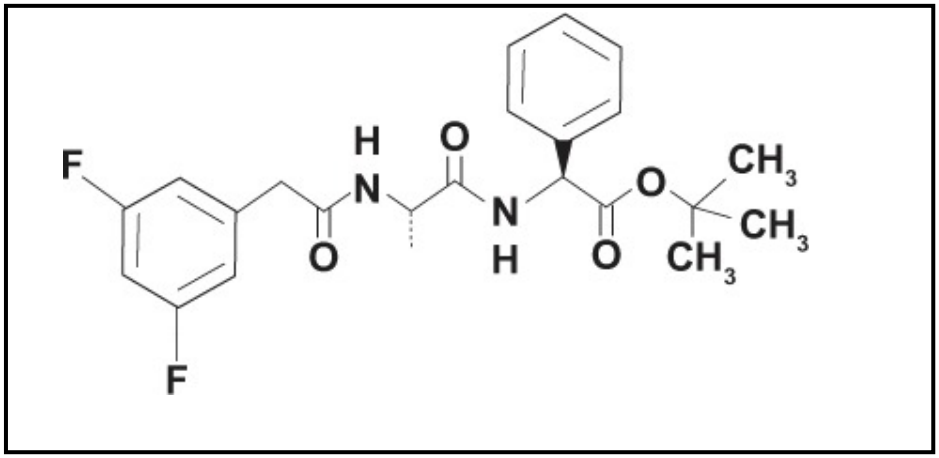


Figure 9: Gamma-secretase inhibitor (DAPT)

The chemical structure of Gamma-secretase inhibitor DAPT.DAPT is also known asN-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester, GSI IX or compound 3 (64).

GSIs can be divided into three main classes : peptides, azepines and sulfonamides. Depending on the structure and function, GSIs can be categorised into two main categories 1) GSIs that mimic and competitively bind to the catalytic active site of Presilins present in gamma-secretase complex. For example DAPT, N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester also commonly known as GSI IX or compound 3 (Figure 9). 2) DAPT potentiated the apoptotic effects in breast cancer cells. DAPT remains to be the start point of GSI mediated therapy as several other effective GSIs have been developed from DAPT (62). These GSIs bind to the interface of the gamma-secretase complex dimer. Forexample Tetarlin imidazole is a GSI that is in Phase I clinical trial of advanced breast cancer.

Treatment	Trail type	Tumor
MK0752	Phase I	Advanced solid tumor
MK0572	Phase I	Normal
MK0752+docetaxel	Phase I/II	Breast cancer
MK0752+gemicitabine	Phase I/II	Pancreatic cancer
RO4929097	Phase II	Renal cell carcinoma
RO4929097	Phase II	Pancreatic cancer
RO4929097	Phase II	Colorectal cancer
RO4929097	Phase II	Non small cell lung cancer
RO4929097	Phase I	Advanced solid tumor
RO4929097	Phase I	Advanced solid tumor
RO4929097+ Capecitabine	Phase I	Refractory solid tumor
RO4929097+ Gemcitabine	Phase I	Advanced solid tumor
RO4929097+ temsirolimus	Phase I	Advanced solid tumor

Table 1: GSIs undergoing clinical trial for various cancer treatments.

Single agent or combinational use of GSIs currently undergoing Phase I\II clinical trials in advanced cancer (64).

Both the GSIs were tested for Notch activity showed decrease tumour cell migration and also downregulation of common Notch HCC etc (64). GSI therapy is usually associated with nominal gastrointestinal side effects. Thus inhibition of Notch signaling via GSI IX to treat Notch active cancers could be of intense therapeutic interest in the foreseeable future (35, 64).

Aim of the study

2. Aim of the study

Gastric cancer is the third leading cause of cancer related death worldwide.Systemic treatment modalities have improved, but the median survival rate is still limited and the 5 year survival rate is less than 20%. Therefore, evolution of new treatment options are of urgent need in the field of gastric cancer. Cancer stem cells (CSC) have been identified in various cancers and are also known to be important in the occurence and metastasis of gastric cancer. CSCs are responsible for chemotherapy resistance and therefore a target of anti-tumour therapies. Therefore, in this present study we tried to specifically target the CSC population in gastric cancer using cell surface markers mainly CD44. Notch and wnt-beta-catenin pathways are also known to be the two main pathways involved in maintenance of CSC. Therefore, using GSI IX we mainly focused on concomitant inhibition of both Notch and wnt betacatenin pathways in the $CD44^{+}$ population of gastric CSCs. Moreover, we also analysed the prognostic value of CD44 and Hes1 double positivity for gastric cancer.Thus, targeting of CD44 by GSI can evolve as an effective, potential therapeutic approach to treat patients with gastric cancer. Analysis of Hes1 and CD44 might help to monitor treatment outcome of gastric cancer as a new biomarker and also to predict prognosis of gastric cancer in this group of patients.

Materials and Methods

3. Material and Methods

3.1 Material

3.1.1 Expendable items

Cover slip	Menzel-Gläser, Braunschweig
Freezing tube	Sarstedt, Nürnberg
Microscope slides	Menzel-Gläser, Braunschweig
Transfer membrane	PerkinElmer, Rodgau
Hyperfilm TM ECL	Amersham Bioscience, Braunschweig
Whatman	Omnilab, Elbingeröder
Centrifuge tubes	Beckmann, Palo Alto
Dialysis tubing	Pierce, Rockford
6 wells plate	Sarstedt, Nürnberg
12 wells plate	Sarstedt, Nürnberg
96 wells plate	Sarstedt, Nürnberg
Cell culture dishes 1000x15 mm	Sarstedt, Nürnberg
Cell culture dishes 60x15 mm	Greiner Bio-One, Frickenhausen
Micro tube 1,5 ml	Sarstedt, Nürnberg
Filter Tips 0,1-10 ul	Sarstedt, Nürnberg
Filter tips 20 ul	Sarstedt, Nürnberg
Filter tips 200 ul	Sarstedt, Nürnberg
Filter tips 1000 ul	Greiner, Solingen
15 ml tube	Sarstedt, Nürnberg
50 ml tube	Sarstedt, Nürnberg

2.1.2 Equipments

Agarose Gelelectrophoresis systems	Bio-RAD, München
Centrifuge 5415 D	Heraeus, Osterode
Centrifuge Mikro 220 R cooled	Heraeus, Osterode
Megafuge 1.0	Hettich, Tuttlingen
Centrifuge “Rotina 38R”	Beckman GmbH, Düsseldorf
Centrifuge “L8-55M”	GFL, Burgwedel
Chaker vibramax 110	Heidolph, Kelheim

Clean Bench	Hera Safe, Kendro, Osterode
Easypet 4420	Sartorius, Göttingen
Electrophoresis power supply	Leica DM5000, Leica, Wetzlar
Fluorescence microscope	Bio-RAD, München
Gel chambers for proteins	Bio-RAD, München
Heater	Heraeus,Instruments GmbH, Osterode
Incubator	Heidolph, Kelheim
Magnetic stirrers	Bio-RAD, München
MicroPulser ^{TM}	Tecan Deutschland GmbH, Crailsheim
Microtiterplate luminometer	Bauknecht, Stuttgart
Microwave	Bio-RAD, München
Mini-Protein Electrophoresis System	Bio-RAD, München
Mini Trans-Blot cell	Bio-RAD, München
Mixer 5432	Eppendorf, Hamburg
Multipette® plus	InoLab, Weilheim
pH-meter	Eppendorf, Hamburg
Photometer	Eppendorf, Hamburg
Pipette	Hirschmann, Eberstadt
Pipetman	Bio-RAD, München
Power supplies	Gilson, Villiers le Bel
Sonificator UP 200H	Bio-RAD, München
Thermo cycler	Hielscher, Stahnsdorf
Thermomixer 5436	MWG-Biotech AG, Ebersberg
ThermoStat plus	Eppendorf, Hamburg
Vortex-Genie	Eppendorf, Hamburg
Water baths	Janke & Kunkel, Staufen

3.1.3 Softwares.

Microsoft Excel 2007 (Microsoft Inc., Remond, Washington)

Microsoft Word 2007 (Microsoft Inc., Remond, Washington)

FlowJo Version 7.3 (Tree Star Inc., Ashland)

BD Cell Quest Pro^{TM} (BD Biosciences, Mississauga)

ImageJ 1.42 (Free Software Foundation, Inc., Boston)

Adobe Photoshop 7.0 (Adobe Systems, San Jose)

MacVector Version 10.1 (MacVector Inc, Cambridge)

3.1.4 Chemicals.

Acetic acid	Baker, Griesheim
Acetone	Merck, Darmstadt
Acrylamid-solution (30%) Mi	AppliChem, Darmstadt
37,5:1	AppliChem, Darmstadt
Annexin V conjugates	Bioline, Luckenwalde
Agar	Bioline, Luckenwalde
Agarose	AppliChem, Darmstadt
Ammoniumacetate	AppliChem, Darmstadt
Ammoniumsulphate	Vector, Eching
Ampicillin	Sigma-Aldrich, Schnelldorf
Antigen unmasking solution	Becton Dickinson, Heidelberg
Antifoam	Sigma-Aldrich, Schnelldorf
BactoTM peptone	Roche, Mannheim
Benzamidine	AppliChem, Darmstadt
ß-Glycerophosphate	AppliChem, Darmstadt
ß-Mercaptoethanol	AppliChem, Darmstadt
Bromophenol blue	Merck, Darmstadt
Boric acid	Sigma-Aldrich, Schnelldorf
Bovine Serum Albumin	AppliChem, Darmstadt
Calcium chloride	AppliChem, Darmstadt
Caesium chloride 99%	Vector, Eching
DAB substrate Kit	AppliChem, Darmstadt
DEPC	Fermentas, St. Leon-Rot
dNTP	AppliChem, Darmstadt
EDTA	AppliChem, Darmstadt
EGTA	Meck, Darmstadt
Ethanol	Sigma-Aldrich, Schnelldorf
Ethanolamine	AppliChem, Darmstadt
Ethidiumbromide	Biochrome, Berlin
FCS	Calbiochem, Darmstadt
Fibronectin	GE Healthcare, Darmstadt

Ficoll-pague TM plus	Merck, Darmstadt
Formaldehyde solution min.	Sigma-Aldrich, Schnelldorf
37% free from acid	AppliChem, Darmstadt
Gelatine	Invitrogen, Karlsruhe
Glucose	AppliChem, Darmstadt
Glutamax	GE Healthcare, München
Glycine	Sigma-Aldrich, Schnelldorf
Gamma secretase inhibitor	Merk, Darmstadt
(GSI)	Sigma-Aldrich, Schnelldorf
Guanidine thiocyanate	AppliChem, Darmstadt
HEPS	Sigma-Aldrich, Schnelldorf
30% H_2O_2	Qiagen, Hilden
Hot start DNA Polymerase	Qiagen, Hilden
Hiperfect transfection reagent	Calbiochem, Darmstadt
Hyaluronic acid	AppliChem, Darmstadt
Imidazole	Merck, Darmstadt
Isopropanol	AppliChem, Darmstadt
Kanamycinesulfate	Sigma-Aldrich, Schnelldorf
Lauroylsarcosine	Sigma-Aldrich, Schnelldorf
Luminol min 97%, HPLC	AppliChem, Darmstadt
Magnesium chloride	AppliChem, Darmstadt
Methanol	Vector, Eching
Mounting Medium	Roche, Mannheim
MTT assay WST-1	AppliChem, Darmstadt
Sodium	AppliChem, Darmstadt
Sodium chloride	BD Difico, Heidelberg
Noble Agar	AppliChem, Darmstadt
Nonfat dried milk powder	AppliChem, Darmstadt
Nonidet P40	Vector, Eching
Normal Horse Serum	Merck-Schuchard, Darmstadt
p- Cumaric acid	Invitrogen, Karlsruhe
Penicillin-Streptomycin solution	AppliChem, Darmstadt
Phenol	PfuTurbo DNA polymerase, Dresden
Polyethylenimine	Roth, Karlsruhe

Ponceau S solution	Sigma-Aldrich, Schnelldorf
Potassium acetate	AppliChem, Darmstadt
Potassium chloride	AppliChem, Darmstadt
Potassium dihydrogen phosphate	AppliChem, Darmstadt
Prolong® gold antifade reagent	Merck, Darmstadt
Propidium iodide	Invitrogen, Karlsruhe
Proteinase K	Fluka, Steinheim
Restriction enzymes	AppliChem, Darmstadt
SDS	NEB, Fermentas
Sodium carbonate	AppliChem, Darmstadt
Sodium chloride	AppliChem, Darmstadt
Sodiumdihydrogenphosphate	AppliChem, Darmstadt
Sodium floride	AppliChem, Darmstadt
Sodium pyrophosphate	AppliChem, Darmstadt
Talon metal affinity resins	Sigma-Aldrich, Schnelldorf
TEMED	Becton Dickinson, Heidelberg
Thymidine 99-100%	AppliChem, Darmstadt
Thermo Pool buffer	Sigma-Aldrich, Schnelldorf
Tris	Biolabs, Frankfurt
Triton X 100	AppliChem, Darmstadt
Trypsin/EDTA solution	AppliChem, Darmstadt
Trypton	Biochrom, Berlin
Tunicamycin	AppliChem, Darmstadt
Tween-20	Sigma-Aldrich, Schnelldorf
Urea	AppliChem, Darmstadt
Vectastain Elite ABC-Peroxidase Kits	AppliChem, Darmstadt
X-Gal for microbiology BC	Vector, Eching Peqlab, Erlangen
Xylenecyanol	AppliChem, Darmstadt

3.1.5 Buffers and solutions

3.1.5.1 Buffers for the cell culture

10X PBS

Constituent	Volume
Nacl	137mM
KCL	2,7mM
Na_2HPO4	100mM
KH_2PO4	2mM

Table 2: Constituents of 10X PBS

The pH was adjusted to 7,4. PBS was sterilised by autoclaving.

Annexin-binding buffer pH 7,4

Constituent	Volume
HEPS	10mM
Nacl	140mM
$Cacl_2$	2,5mM

Table 3: Constituents of Annexin-binding buffer

Proteinase K buffer

Constituent	Volume
Tris pH 8,5	100mM
EDTA	5mM
SDS	0,2%
Nacl	200mM
ddH_2O	100ml

Table 4: Constituents of Proteinase K buffer

3.1.5.2 Buffers and solutions for protein extraction and analysis

3.1.5.2.1 Protein extraction

RIPA

Constituent	Volume
Tris pH 8,0	50mM
Nacl	80mM

NaF	50mM
$Na_4P_2O_7$	20mM
EDTA	1mM
EGTA	1mM
NP-40	1%
DOC	1%
SDS	0,1%

Table 5: Constituents of RIPA buffer

NP40

Constituent	Volume
Tris pH 7,5	50mM
Nacl	150mM
NaF	50mM
$Na_4P_2O_7$	20mM
EDTA	1mM
ß Glycerolphosphate	10mM

Table 6: Constituents of NP40

4XSB

Constituent	Volume
Tris pH 6,8	0,25 M
SDS	8%
Glycerol	40%
ß-Mercaptoethanol	10%
Bromophenol blue	0,05%

Table 7: Constituents of 4XSB

Protease and phosphatase inhibitors (for 10 ml buffer)

Name of the inhibitor	Type	Final concentration
Vanadat	Tyrosine-phosphatase	100µM
Leupeptin	Aspartic protease	0,5 ng/ml
Leupeptin	Serine-cystein protease	0,15mM
Benzamidin	Trypsin, thrombin, Plasmin	0,5%

Aprotonin	Trypsin, chymotrypsin, kallikerin	0,5mM
PMSF	Serine-protease	0,5mM

Table 8: List of the used protease and phosphatase inhibitors

3.1.5.2.2- Protein analysis

Separating Gel

% Acrylamide	7,5 %	10 %	12 %	14 %	15 %
Acrylamide	2,5 ml	4,1 ml	3,4 ml	2,7 ml	**2,45 ml**
Tris pH 8,0	2,5 ml	2,5 ml	2,5 ml	2,5 ml	**2,5 ml**
H_2O	4,85 ml	4,1 ml	3,4 ml	2,7 ml	**2,45 ml**
10 % SDS	100 ul	100 ul	100 ul	100 ul	**100 ul**
TEMED	15 ul	15 ul	15 ul	15 ul	**15 ul**
10 % APS	**105 ul**	**105 ul**	**105 ul**	**105 ul**	**105 ul**

Table 9: Constituents of the used separating gels

Stacking gel

Constituent	Volume
Acrylamide	1,13ml
Tris 6,8 pH	1,75ml
ddH_2O	3,2ml
10% SDS	70ul
TEMED	7µl
10% APS	70µl

Table 10: Constituents of the used stacking gel

SDS running buffer (10x)

Constituent	Volume
Tris pH 8,3	250mM
SDS	1%
Glycine	1,92M

Table 11: Constituents of SDS running buffer (10X).

Western transfer buffer (10x)

Constituent	Volume
Tris	250mM
Glycine	1,92M

Table 12: Constituents of western transfer buffer (10X).

TNT western blot washing buffer (10x)

Constituent	Volume
Nacl	1,5M
Tris pH 7,5	100mM
Tween-20	0,5%

Table 13: Constituents of western blot washing buffer (10X).

Blocking solution

Milk powder, nonfat 5 % in TNT

ECL solution

Solution 1

Constituent	Concentration
Luminol	250 mM
p-cumaric acid	0,4 mM
Tris pH 8,5	0,1 M

Table 14: Constituents of ECL solution (solution 1)

Solution 2

Constituent	Concentration
Tris pH 8.5	0.1M
30% H_2O_2	0.061%

Table 15: Constituents of ECL solution (solution 2)

2.1.6.2 Solutions used for immunohistochemistry

Antigen Unmasking Solution

Antigen unmasking solution 1:100 diluted in dH_2O

Blocking Solution (in PBS)

Constituent	Volume
Normal Horse serum	5%
Triton X-100	0.3%

Table 16: Constituents of blocking solution for immnuhistochemistry

Acid Rinse Solution

Constituent	Volume
Glacial Acetic acid	2%

Table 17: Constituents of acid rinse solution for immnuhistochemistry

Ethanol

100%, 95%, 70%, 40%

3.2 Methods

3.2.1 Cell culture

Human gastric cancer cell lines (2313287, KATO III, SNU1, NCI-N87 MKN 45) were obtained from Derek Zieker (Wiesbaden, Germany) and Kuang Hung Cheng (Kaohsiung, Taiwan). Human pancreatic cancer cell line KP3 was obtained from Nabeel Bardeesy (Boston, USA). MKN45 was cultured in RPMI 1640 + Glutamax (Invitrogen, Karlsruhe, Germany) supplemented with 20% FCS (Biochrom, Germany) and 100 U/ml penicillin/streptomycin (Invitrogen,Germany) at 37°C in 5 % CO_2. KP3, 2313287, SNU, NCI-N87 were cultured under same conditions, supplemented with 10% FCS 100 U/ml penicillin/streptomycin (Invitrogen, Germany) at 37°C in 5 % CO_2. KATO III cells were cultured in DMEM (Invitrogen, Karlsruhe, Germany) supplemented with 10% FCS and 100U/ml penicillin/streptomycin (Invitrogen, Germany) at 37°C in 5 % CO_2. Cells were checked for authenticity every six months by growth curve analysis, morphology check by microscope. Cells were regularly

checked for mycoplasma contamination. Adherent cells (2313287, SNU1,NCI-N87,KP3) were subcultured in every two days on reaching 70-80% confluency. For subculturing the adherent cells, the cells were trypsinised with 1 ml of trypsin at 37°C incubator for few minutes to collect the adherent cells and were transferred into new cell culture plates containing fresh medium. Suspension cells (KATO III, MKN45) were collected as a pellet by centrifugation (1000 rpm for 5 minutes), resuspended in fresh medium and transferred into new culture plates. Cells were cultured for a limited passage. Cells were frozen for long term storage, in order to freeze the cells both adherent and suspension cells were collected by centrifugation at 1000 rpm for 5 minutes . The pellet was resuspended in freezing medium ($1x10^6$ cells/ml) (FuCS with 10% DMSO), DMSO was used as a cryoprotective agent. The freezing medium was prepared fresh during each time of freezing. Frozen cell aliquots were stored at -80°C.

3.2.2 Drug preparation and *in vitro* treatment

3.2.2.1 Drug preparation

Gamma secretase inhbitior IX , GSI IX (Calbiochem, EMD Chemicals) is provided as a 200mg light protective lyophillised powder from the company. GSI IX was prepared as 100mM stock in dimethylsulfoxide, DMSO (AppliChem, Darmstadt) and stored in light protective vials at -20°C.

3.2.2.2 Drug treatment *in vitro*

Human gastric cancer cell MKN45 were treated with dimethylsulfoxide, DMSO (AppliChem, Darmstadt) or GSI IX (Calbiochem, EMD Chemicals) in different concentrations (2.5µM, 5µM and 15µM) and were analyzed after 48hours and 96hours .

3.2.3 Apoptosis Assay

In order to detect the induction of apoptosis in MKKN45 cells by GSI IX cells were subjected to apoptosis analysis using Annexin V\PI staining kit (Annexin V FITC Apoptosis Detection Kit I, BD Biosciences). For apoptosis cells were seeded in 6-well

tissue culture plates at a density of $1x10^5$cells per well and were incubated over night at 37^0C. These cells were further treated with DMSO or GSI (2.5, 5 and 15µM) for 48 and 96 hours. The medium was changed every two days. At 48 and 96 hours, the cells were harvested. The cells were washed with 1×PBS and trypsinised with 1ml trypsin for few minutes at 37°C incubator. The cells were collected after trypsinisation by centrifugation for 5 min at 1000 rpm. The cell pellet was resuspended with 1 ml 1×PBS and centrifuged for another 5 minutes. The supernatant was discarded again, the cell pellet was suspended with 1ml 1x Binding Buffer (FITC Annexin V Apoptosis Detection Kit I, BD Biosciences) on ice. The untreated cells were divided into three tubes (Annexin V-control, PI-control and unlabeled control). 5µl Annexin V and 10µl PI (Annexin V FITC Apoptosis Detection Kit I, BD Biosciences) were added into the corresponding tubes. The samples were incubated for 15 minutes on ice and were further subjected to FACS analysis. The signal was detected using FACS calibur flow cytometer (BD, Heidelberg) and analyzed using FlowJo Version 7.6 software (Tree Star Inc., Ashland, USA). Characterized proportion of apoptotic cells in the untreated cell population compared to the total cell population of apoptotic cells was subtracted to obtain the respective proportion of the cells undergoing apoptosis, under each experimental condition. Living cells are thus Annexin V and PI negative. Early apoptotic cells are Annexin V positive and PI negative. Late apoptotic cells or dead cells are both Annexin V and PI positive. The quantitative measurement of the percentage of apoptotic cells is usually performed in the whole population following the manufacturer's protocol (Annexin V FITC Apoptosis Detection Kit I, BD Biosciences)

3.2.4 Cell proliferation assay

In order to measure the effect of GSI on cell proliferation, $CD44^+$ MKN45 cells were plated at a concentration of 1000 cells/ ml in a 96 well plate overnight. Cells were treated with DMSO, different concentrations of GSI (Merck KGaA) (2.5µM, 5µM and15µM) or 5-fluorouracil (5FU) (Sigma Aldrich, USA) (15µM) for 4 days. At each time point starting from 24 hours to 96 hours, 10 µL WST-1 reagents (Roche Diagnostics, Germany) was added to each well and incubated for 2 hours at 37°C light protected. The absorbance was detected at a wavelength of 492 nm with reference wavelength of 650 nm. Light microscopic pictures (10X magnification) were

taken for cells treated with DMSO or GSI on the final day (96 hours) of the experiment. The relative cell number was determined by plotting the optical density (OD) of cells under GSI treatment compared to the DMSO treated cells for each time points.

3.2.5 Migration Assay

Migration analysis was done in order to analyse the effect of GSI IX treatment on migration of cells. $CD44^{+}$MKN45 cells were seeded in a 6-well plate and left to reach 80% confluency. Initially, cells were starved for 24hours in media containing 2% FCS. Then the sorted cells were further incubated for 48hours in the starvation media containing either the control with DMSO, different concentrations of GSI (2.5µM, 5µM and15µM). Afterwards a scratch was done using a white tip for each treatment. Then cells were washed with PBS and photographed using Leica DMI 6000 B microscope (Leica). Cells were incubated for an additional 24hours after which the photographs were taken for the wounded area. The migrating cells were calculated according to the following formula:

$$\text{Migration Index} = \frac{\text{Width of the wound at 0h - Width of the wound at 24h}}{\text{Width of the wound at time 0h}} \times 100$$

For each treatment, the difference in wound diameter between time 0hour and 24hours was determined in relation to the 0hour reading. Since each wound has several measuring points, an average was determined for calculating the migration index.

3.2.6 Invasion Assay

In order to perform an invasion assay the invasion chambers (BD Biosciences, UK) were acclaimatized to room temperature and rehydrated serum-free medium for 2 hours at 37° C incubator were $CD44^{+}$MKN45 cells ($2.5x10^{5}$ cells / 2ml) were seeded in serum free media into each well of the 6-well BD BioCoat™ Matrigel™ Invasion Chamber (BD Biosciences). The cells in the inserts were simultaneously treated with

GSI (2.5µM, 5µM and15µM) and the DMSO control. The inserts were placed into the BD Falcon TC Companion Plate containing 10% FCS and incubated for 48 hours in a humidified tissue culture incubator, at 37°C, 5% CO_2 atmosphere. Then the invading cells were fixed with 100% methanol (AppliChem, Darmstadt) and stained with 1% toluidine blue (Sigma-Aldrich, St. Louis, USA) in 1% borax (Sigma-Aldrich, St. Louis, USA). Cells were then counted under the microscope (Leica DM 5000 B, Leica, Germany). The calculation of the invading cells was done according to the BD protocol where:

$$\text{Invasion Index} = \frac{\%\ \text{Invasion Test Cell}}{\%\ \text{Invasion Control Cell}}$$

3.2.7 Western blot

3.2.7.1 Protein quantification

The protein quantification was carried out according to manufacturer's instructions for Protein Assay kit (BioRad). 5µl of each protein sample was added as triplicates to a 96 well plate. 20µl of reagent S was added to an Eppendorf containing 1ml of reagent A. 25µl of Reagent (A+S) was added to each well. 200µl of Reagent B was added to each well and the plate was incubated for 20 minutes light protected at room temperature. The absorbance (650nm) was measured with the Micro plate-Reader Multiskan Plus (Titertek-Berthold, Pforzheim). The mean value of the protein sample from the triplicate well was used for analysis. The amount of cell lysate in 1µl calculated as follows: 100 / ((x-0.0627) / 0,136). The net volume of the protein was standardized to 100µl containing 25µl of 4X loading dye.

3.2.7.2 Sample preparation and Western blotting

The cell were collected and transferred to 15ml test tubes and centrifuged for 5 min at 1000rpm. The supernatant was discarded, the cell pellet was resuspended with 1ml ice cold 1×PBS and centrifuged again. The supernatant was discarded and the cell pellets were incubated in 200µl Western lysis buffer buffer supplemented by protease and phosphatase inhibitors for 30minutes on ice. The samples were sonificated on ice (15 seconds per sample). Thereafter, the suspensions were centrifuged for 25 minutes at 13000 rpm and 4°C. The samples were stored at -20° C until used.

$CD44^{+}$MKN45 cells were cultured with DMSO or GSI (2.5µM, 5µM and 15µM). Harvested cells were lysed in lysis buffer containing 20mM Tris, 150mM NaCl, 1mM EDTA, 1mM EGTA, 1% Triton X-100 and protease and phosphatase inhibitor (Protease Inhibitor Cocktail Tablets, Roche). The concentration of extracted protein was determined using DC protein assay kit (Biorad) following manufacturer's instruction as mentioned above. Samples were denatured in gel loading buffer by heating at 95°C for 5 minutes. For western blotting the cell lysates were loaded at a protein concentration of 30µg per well. Gel electrophoreses (12% acrylamide gels) was performed (Biorad). Initially the electrophoretic separation was conducted at 80V until the separating gel was reached, then the voltage was increased to 120V. The PVDF membrane was briefly incubated in 100% methanol for activation followed by equilibration of the membrane in 1x transfer buffer for few minutes. The gel was then placed into the western blot cassette (the order of the western blot sandwich: sponge-filter paper- PDVF membrane- gel-sponge-filter paper). The cassette was placed into the western blot apparatus and run at 100V for 1 hour. The membranes were blocked using 5% dried milk (AppliChem) for 30 minutes at room temperature. The membrane was subsequently washed thrice with 1X TNT, 10 minutes for each wash. Then the membrane was probed with primary antibodies overnight in 1X TNT at 4^{0}C. After overnight incubation of the primary antibody, the membrane was washed thrice with PBS (5 minutes each wash). After washing the secondary antibody was added in 1X TNT and incubated for 1 hour at room temperature. After another three washes with 1xTNT wash buffer the PVDF membrane were exposed to Amersham Hyperfilm ECL (GE Healthcare Limited Buckinghamshire), ECL-films.

Target	Working dilution	Source	Manufacturer
Anti-E-Cadherin Rabbit mAb	1:1000	Rabbit	Cell signaling
Anti-N-cadherin rabbit monoclonal	1:50000	Rabbit	Cell signaling
Monoclonal anti-Actin (C-2)	1:10000	Mouse	Santa Cruz Biotechnology
Anti-CD44 antibody	1:1000	Mouse	Cell signaling
Hes1 antibody	1: 500	Mouse	Santa Cruz Biotechnology
p-AKT	1:500	Rabbit	Cell signaling
Beta-catenin	1:1000	Rabbit	Cell signaling
P-Stat3(Tyr 705)	1:1000	Rabbit	Cell signaling
Rac1	1:500	Rabbit	Cytoskeleton Inc.,
PARP	1:1000	Rabbit	Cell signaling
P21	1:500	Rabbit	Cell signaling

MAPK (Erk 1/2)	1:500	Rabbit	Cell signaling
STAT3	1:500	Rabbit	Cell signaling
c-Myc	1:1000	Rabbit	Santa Cruz Biotechnology
Wnt 5a\b	1:1000	Rabbit	Cell signaling
Notch1	1:1000	Rabbit	Abcam
Notch2	1:1000	Rabbit	Abcam
Notch3	1:1000	Rabbit	Abcam
Axin2	1:1000	Rabbit	Cell signaling
APC	1:1000	Rabbit	Cell signaling
LPR6	1:1000	Rabbit	Cell signaling
Anti-beta actin	1:1000	Mouse	Cell signaling
Anti-alpha tubulin	1:1000	Mouse	Abcam

Table 18: List of primary antibodies used for western blot

Target	Working dilution	Manufacturer
Anti-mouse IgG peroxidase conjugated	1:10000	Amersham
Anti-rabbit IgG peroxidase conjugated	1:10000	Amersham
Mouse TrueBlot™HRP-conjugated anti-mouse IgG (clone eB144/7A7)	1:15000	Bioscience

Table 19: List of secondary antibodies used for western blot

3.2.8 Tumour sphere assay

$CD44^{+}$MKN45 cells were resususpended in serum-free RPMI 1640 culture medium containing 1% N2 supplement, 2% B27 supplement, 1% antibotic-antimycotic (Invitrogen) 20 ng/ml human FGF-2 (Sigma, Saint Louis and 100 ng/ml EGF (Invitrogen) containing either the control with DMSO or different concentrations of GSI (2.5μM, 5μM and 15μM), and plated in 96-well ultra-low attachment plates (Corning, USA) at 2,000 cells per well. 7–10 days later, plates were analyzed for tumoursphere formation and were quantified using an inverted microscope (Olympus) at 100X, 200Xmagnification. The size of the tumoursphere formed under each condition was also considered during quantification.

3.2.9 Immunohistochemistry

Tissue sections were fixed in 4% formalin (Sigma-Aldrich) overnight, stored in PBS and embedded in paraffin. For immunohistochemistry slides were deparaffinised using an oven at 550ºC overnight (GE Healthcare Limited) and rehydrated in decreasing ethanol concentrations (6 min to 100%, 95%, 75%, 40% ethanol) (Merck) and then rinsed three times for 3-5 minutes with 1xPBS. Antigen retrieval was performed by heating the slides in pressure cooker with Antigen Unmasking Solution (Vector Laboratories, Inc.) The slides were then washed in PBS and incubated for 10 minutes in 1% H_2O_2 (Sigma-Aldrich, USA), rinsed with PBS, and incubated 1 hour in 100µl of blocking solution (5% normal serum þ 0.3% Triton X-100, Vector Laboratories, Inc.) at room temperature protected from light. Hybridization with the 100µl primary antibody in 1X PBS was carried out overnight at 4°C. The next day the sections were washed three times for 3 minutes each in 1×PBS then 100µl of secondary antibody was incubated for 1 hour at room temperature, light protected. The sections were then rinsed three times for 3 minutes with 1 × PBS, 100 µl of ABC reagent (Vectastain ABC Kit, Vector Laboratories) was added to the tissue and incubated for one hour at room temperature followed by three times in 1 × PBS rinse subsequently. 300 ul freshly prepared Vector DAB substrates (Peroxidase Substrate Kit DAB, Vector Laboratories) were applied to the tissues and waited up to 3 minutes until a desired DAB background was obtained. Subsequently, the slides were immersed in distilled water to stop the DAB reaction. This was followed by counterstaining with hematoxylin for 1 minute and subsequent rinsing with water. Slides were counterstained with hematoxylin (Merck) and dehydrated in 40%, 70%, 90%, and 100% ethanol. Finally, slides were cleared with Rhothistol (Roth) and mounted with Permount Toluene Solution (Fisher Scientific).

Staining intensity was graded as negative staining (0); defined as missing nuclear and/or cytoplasmic staining even when using high amplification (40x). Weak staining (1); defined as nuclear and/or cytoplasmic staining visible at high magnification (40x) only. Moderate staining (2) is defined as nuclear and/or cytoplasmic staining visible at medium magnification (10x-20x amplification). Strong staining (3) is defined and/or cytoplasmic staining already visible at low magnification (2.5x-5x amplification). The intensity of staining was scored independently by two investigators. Ten high power

fields were randomly selected from each slide for scoring expression of CD44 and HES1 staining. Positive staining was defined as grade 2-3 and negative as 0-1.

Target	Working dilution	Source	Manufacturer
Anti CD44 antibody	1:100	Rabbit	Novus Biotechnology
Hes1 antibody	1:100	Rabbit	Abcam
NCL – ki67P	1:100	Rabbit	Novocastra, Leica

Table 20: Primary antibody for immunohistochemistry.

Target	Working dilution	Manufacturer
Anti-Rabbit IgG (H+L) biotinylated	1:200	Vector laboratories

Table 21: Secondary antibody for immunohistochemistry.

3.2.10 Immunofluorescence

$CD44^{+}$MKN45 cells were cultivated on cover slips prior to immunofluorescence. The cover slips were washed with 1XPBS three times and were fixed with 4% formaldehyde. Then the cells were washed with PBS three times again and permeabilized with 1% Triton-X for 5 minutes. Cells were blocked with 10% Bovine Serum Albumin (BSA, Sigma) for 30 minutes and afterwards incubated at 37°C for 1 hour with the 100µl of primary antibody in 1xPBS . After primary antibody incubation the cells were washed three times in PBS. This was followed by incubation of the cells with 100µl of secondary antibody for 30 min at room temperature in dark. Cells were washed with 1X PBS and were stained with 4,6-diamidino-2-phenylindole (DAPI, Sigma) in order to reveal cell nuclei. Finally, stained cells were analyzed using a fluorescence microscope (Leica DMI 6000 B microscope) to measure the CD44 and HES1 expression.

Quantification for TMAs was performed using ImageJ freeware and expressed per mm² of total tissue area. For the analysis of CD44, Hes1, Hes1 + CD44 staining, the positive stained cells where chosen from six randomly selected areas for each tumour (using a scale of 100µM). Staining intensity was graded as negative staining (0); defined as missing staining (40x). Weak staining (1); defined as staining visible at high magnification (40x) only. Moderate staining (2) is defined as staining visible at medium magnification (10x-20x amplification). Strong staining (3) is defined already visible at low magnification (2.5x-5x amplification). The staining intensity was calculated as a percentage of total tissue area. The intensity of staining was scored independently by two investigators. Positive staining was defined as grade 2-3 and negative as 0-1.

Target	Working dilution	Source	Manufacturer
Anti CD44 antibody	1:100	Rabbit	Abcam
Hes1 antibody	1:100	Rabbit	Abcam

Table 22: Primary antibody for immunofluorescence/Flow cytometry.

Target	Working dilution	Manufacturer
Anti mouse FITC	1: 200	Dianova
Anti Rabbit Alexa Flour 594	1:200	Life Technologies

Table 23: Secondary antibody for immunohistochemistry.

3.2.11 CD44 cell sorting and flow cytometry (FACS)

3.2.11.1 Sample preparation and sorting.

CD44 populations were sorted from human gastric cancer cell line MKN45. Cells were washed in ice cold 1X PBS at 500 rpm for 5minutes. This was followed by blocking 100 µl of blocking buffer (5% BSA/PBS) for 30 minutes on ice. Cells were centrifuged at 500 rpm for 5 minutes at 4°C. Cells were then incubated with 100µl of primary antibody in 1 mM EDTA/1%BSA/PBS for 25 minutes on ice. Cells were resuspended with 100µl of ice-cold 1 mM EDTA/1%BSA/PBS and centrifuged at 500

rpm for 5minutes. After centrifugation the cells were stained with 100µl of secondary flurochrome conjugated antibody for 20 minutes in dark. The stained cells were washed twice with 1 mM EDTA/1%BSA/PBS, centrifuged at 500 rpm for 5 minutes and the cell pellet was suspended in 200 µl of 1mM EDTA/1%BSA/PBS prior to analysis. The stained cells were measured with FACS Calibur (Becton Dickinson Immunocytometry Systems). KP3 cells were used as a positive control as previously published (84).

After preparation of the samples, the cells were subjected to FACS sorting. $CD44^+$ and $CD44^-$ population of MKN45 cells were collected under sterile conditions in different sterile facs tubes. The sorted cells were washed twice with 1XPBS by centrifugation at 500 rpm for 5 minutes at 40ºC and were added to the plates containing complete culture medium (RPMI 1640+Glutamax)supplemented with 20% FCS and penicillin / streptomycin (100 units/ml).

3.2.11.2 *In vivo* handling of CD44 sorted MKN45 cells

For *in vivo* experiments, 6×10^4 sorted cells were resuspended in PBS/Matrigel (mixture 1:1 volume, BD Biosciences) and were injected subcutaneously in to the left and right flank of 6-week-old female nude mice (*NMRI-nu/nu*). For *in vitro* experiments the sorted cells were taken into cell culture and were treated with control (DMSO) or GSI inhibitor (2.5µM, 5µM, 15µM) (Calbiochem). Sorted $CD44^+$ and unsorted cells were used for the following experiments: proliferation assay, migration assay, invasion assay, tumour sphere assay, immunoblotting, apoptosis assay, nuclear cytoplasmic extraction, immunofluorescence staining and siRNA transfection.

3.2.11.3 Isolation of mononuclear cells from blood for FACS analysis

One ml of blood in Heparin was collected from control or GSI treated xenografts from the heart. The blood was further subjected to Ficoll-Plaque PLUS (Amersham Bioscience) density gradient centrifugation at 500rpm for 30 minutes in order for mononuclear cells to be isolated (MNC). Concentrated MNCs were harvested from the interface with the help of a disposable Pasteur pipette by placing the sample on ice. The isolated cells were washed twice in ice-cold PBS, then centrifuged for 30 minutes at 500 rpm. Isolated MNCs were processed for flow cytometric analysis using staining protocol for FACS analysis as mentioned above. In addition, 10ml

blood from healthy donors (n=4) and patients with untreated gastric cancer (n=3) was collected and further processed like mentioned.

CD44 and HES1 detection was routinely performed with the primary antibody omitted to control for background staining. Double-staining (CD44 and Hes1) was compared to parallel cells stained for CD44 or HES1 alone. Cells were routinely sorted twice and reanalyzed for purity (XDP, Beckman-Coulter).

3.2.12 Tumour sphere forming assay

$CD44^{+}$MKN45 cells were suspended in serum-free culture medium DMEM containing 1% N2 supplement, 2% B27 supplement, 1% antibotic-antimycotic (Invitrogen, Germany) 20 ng/ml human FGF-2 (Sigma, Saint Louis, USA), and 100 ng/ml EGF (Invitrogen, Germany) containing either the control with DMSO or different concentrations of GSI IX (2.5µM, 5µM and 15µM), and plated in 96-well ultra-low attachment plates (Corning, USA) at 2000 cells per well. 7–10 days later, plates were checked under the microscope for tumoursphere formation. Tumourspheres were analyzed and quantified using an inverted microscope (Olympus, Germany) at 100X, 200X magnification. The microscopic images of the tumourspheres were taken on the day of quantification. During quantification the difference in size of the tumourspheres between the DMSO control and GSI treated cells were also considered. For each well the average count of the tumourspheres formed were taken into consideration for quantification.

3.2.13 Adhesion assay

Collagen type IV (Bovine, Sigma) and Hyaluronic acid (bovine, Calbiochem) were diluted from stock concentration of (1mg/ml) to (5µg\ml) using 1X PBS. In a 96 well plate 100µl\well of the substrate was added into their respective well in triplicate. The 96 well plate containing the substrates were allowed to settle for 2 hours at room temperature under the culture hood. After 2 hours the substrates were removed from the wells. Each well was washed two times with 200µl of 1XPBS. After washing 200µl of blocking buffer (RPMI 1640+Glutamax containing 10% FCS) was added to the wells and incubated for 30 minutes at $37^{\circ}C$, 5% CO_2 incubator. After blocking the wells were again washed with 200µl of 1XPBS. $5x10^4$ cells/100µl with DMSO or GSI IX (2.5, 5 and 15µM) was added to their respective wells in triplicates. The 96 well

plate was incubated overnight at 37^0C, 5% Co2 incubator. Next day the plate was inverted upside down and incubated at 37^oC, 5% Co_2 for atleast 1.5 hours. After incubation the plate was tilted at an angle of 45^0 and the medium was transferred to a fresh 96 well plate into their respective wells (DMSO or GSI IX) treated. The fresh 96 well plate contains the suspension cells under each condition whereas the other plate contains the adherent cells. Using WST-1 reagent (Roche, Darmstadt) the number of suspension cells and adherent cells in the respective plates under each condition (DMSO or 2.5, 5 and 15µM GSI IX) was calculated and compared.

3.2.14 Nuclear cytoplasmic extraction

1.5x 106 $CD44^+$MKN45 cells were treated with control DMSO or different concentrations of GSI (2.5µM, 5µM and15µM) for 48 and 96 hours and subjected top nuclear cytoplasmic extraction using NE-PER™ Nuclear and Cytoplasmic Extraction kit (Thermo Fisher Scientific, USA) and following the kit instruction manual. The cells were collected for nuclear cytoplasmic extraction in 1xPBS. 100µl of ice cold CER I reagent and 5.5µl of CERII reagent and 50µl of NER reagent (the reagents were provided in the kit) was added to the cell pellet followed by vigorous vortexing of the tube at highest setting for 15 seconds (volume ratio CER I: CER II at 100:5.5 µl). After vortexing the tube was incubated for 15 minutes on ice followed by centrifugation for 5 minutes at 1000 rpm. The supernatant was immediately transferred to a pre chilled tube and stored at -20°C, this tube contains the cytoplasmic fraction. The insoluble pellet was resuspended in 50µl of NER buffer. The sample was vortexed for 5 seconds followed by centrifugation for 5 minutes at 1000 rpm. Immediately after centrifugation the supernatant was transferred to another fresh prechilled tube and stored at -20°C. This tube contains the nuclear fraction. Extracts were stored at -20°C until used.

3.2.15 Small interfering RNA (siRNA) transfection

To knock-down Notch1 and CD44 in MKN45 cells we used synthetic pre designed siRNA for Notch1 (Ambion Thermo Fisher scientific) and CD44 (Ambion Thermo Fisher scientific, USA). MKN45 cells were plated at a concentration of $5x10^3$ cells/ml in 6 well plate and were transfected with transfection reagent containing control siRNA (Ambion Thermo Fisher scientific, USA) at a concentration of 10nM, 25nM, 30nM and 50nM or Hes1/CD44 siRNA for 24hours, 48hours and 72hours using

Hiperfect transfection reagent (Qiagen,USA). The tubes containing siRNA were diluted to working concentration in Hiperfect transfection reagent and were vortexed shortly for five seconds followed by incubation for 15 minutes in room temperature prior to addition to the cells. After incubation the mixture was added drop by drop to the cells. The cells were kept in the incubator at 37^0C according to the time desired (between 48 to 96 hours) The transfection efficiency was judged based on western blot analysis at 48 and 96 hours.

3.2.16 Patient samples and tissue microarray (TMA)

Fresh human tissues were collected from patients who underwent endoscopic procedure at the Medizinische Universitätsklinik Tübingen, Germany. Tissues were representing normal gastric mucosa (N), inflammation (G) and gastric cancer. Tissue microarrays composed of 269 human gastric cancer specimens were obtained from the Institute of Pathology, Medical School Hannover (MHH), Germany.[34] The clinical, pathological and survival data were also obtained from the same patients. Tissue microarrays composed of 269 human gastric cancer specimens were obtained from the Institute of Pathology, MHH, Germany. The clinical, pathological and survival data were also obtained from the same patients (93).

3.2.17 Animals and *Invivo* treatment

For *in vivo* experiments, 6×10^4CD44 sorted cells were resuspended in PBS/Matrigel (mixture 1:1 volume, BD Biosciences) in ice and were injected subcutaneously in to the left and right flank of 6-week-old female nude mice (*NMRI-nu/nu*). *NMRI-nu/nu* female nude mice were obtained from Charles River Laboratories International (Sulzfeld, Germany). In total 10 mice were used for MKN45 xenograft experiments. $CD44^+$ MKN45 cells ($1x10^6$) were subcutaneously injected into each flank for each mouse. Mice were assigned into treatment groups (5 mice per group). Tumour size was measured by caliper and tumour volume was calculated using the following formula: Tumour volume V= [(ð\6) x (Lenght) x ($Width^2$)]. When tumour volume reached a size of $100mm^3$, mice were treated intraperitoneally with GSI (10 mg/kg/body weight) or control (PBS) for four times per week followed by three days rest. The drug was prepared by dissolving in PBS with 10% DMSO. The compound was stored at ambient temperature and resuspended prior to use in order to ensure

that no particles settled at the bottom. Health status and treatment side effects were inspected every day. After 4 weeks of therapy when the control mice tumours reached a size of 1000mm^3 all mice were sacrificed, tumours were snap frozen for protein analysis and fixed in 4% formalin for histology. The weight of the tumours for two groups (control and GSI treated) was also measured. The mice used in this study were maintained in the animal care of Medizinische Universitätsklinik Tübingen (UKT), Germany. All experimental protocols were reviewed and approved by institutional guidelines for animal care of UKT and Baden Württemberg (protocol no: M 11/12).

3.2.18 Statistical analysis

All the experiments were repeated 3 times. Data were presented as mean ± standard deviations (41). The results were analyzed using software Graphpad prism version 5.0 (GraphPad Software, San Diego, CA) and SPSS Version 11.0 (SPSS, Chicago, USA). The tests include one-way ANNOVA analysis of variance, student´s *t*-test along with Bonferroni post test and paired, unpaired t-tests. The overall survival analysis using Kaplan-Meier curves were analysed by log rank test. For all tests, a P value of <0.05 was considered statistically significant.

Results

4. Results

4.1 CD44 and Hes1 are effectively upregulated in both gastric cancer cell lines, human tissues and also show an influence on patient survival.

CD44 is an established cell surface marker for CSCs and is also known to play an important role in gastric cancer progression. Therefore, in order to target these population of tumour cells positive for CD44 known to be the main candidates for therapy resistance,we performed some initial screening.

First we analysed five different gastric cancer cell lines to validate the expression of CD44 by using Flowcytometry. The cell lines analysed for initial screening were MKN45, SNU-1, 2313287, NCI-N87. MKN45 is a human gastric cancer cell line derived from a 62 year old female having poorly differentiated adenocarcinoma (65).SNU (Seoul National University) cell lines are derived from various cancer patients in
Korea since 1982. SNU1 is one of the gastric carcinoma cell lines derived during this study in Korea. (66) For our present study SNU 1 cell line was used. KATO III is a human gastric carcinoma cell line developed *in vitro* from a pleural effussion of a 55 year old male patient (66). NCI-N87 cell line is derived from well-differentiated human gastric carcinoma. This cell line exibhits some true features of gastric epithellium (68).

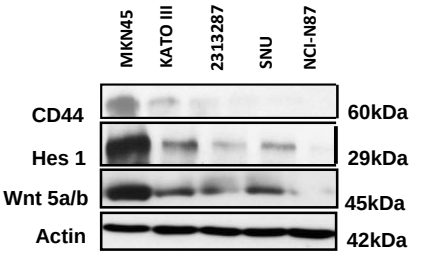
Flow cytometric analysis showed MKN45 -65%, Kato III – 18.4%, 2313287 – 8.7%, SNU – 4.5%. of CD44 positive cells. Amongst this panel of five different gastric cancer cell lines analysed, MKN45 showed the highest population of $CD44^{+}$ cells (65%) as compared to other gastric cancer cell lines. In contrary SNU-1 cell lines showed the lowest percentage (4.5%) of $CD44^{+}$ cells. Since Notch and wnt-beta-catenin pathways are known to be the two most important pathways associated with gastric CSC maintenance, therefore specific targeting of these two pathways in gastric cancer treatment could be of interest. Next we wanted to analyse the expression level or activation of these pathways in our selected MKN45 cells. Therefore we checked the expression level of Hes1 (downstream of Notch pathway) and wnt 5a/b (the non canonical ligand of wnt and is associated with the agressiveness of the tumour) by western blot. We reconfirmed the expression of all

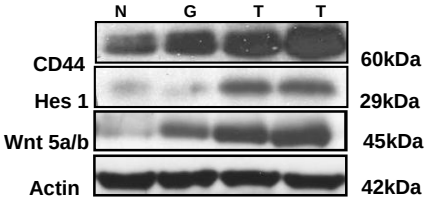
the three targets (CD44, Hes1, wnt 5a/b) in these five cell lines and among all of them in accordance with the FACS data MKN45 showed relatively high expression of

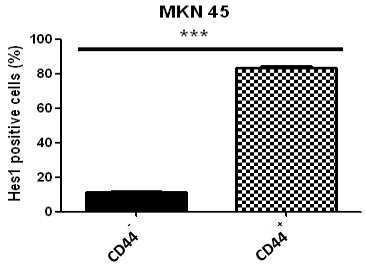
all the targets. (Figue 10 A, B). Thus from the screening we selected MKN45 for our *in vitro* experiments as this cell line showed significant expression of all the three targets (CD44, Hes1 and wnt5a/b).

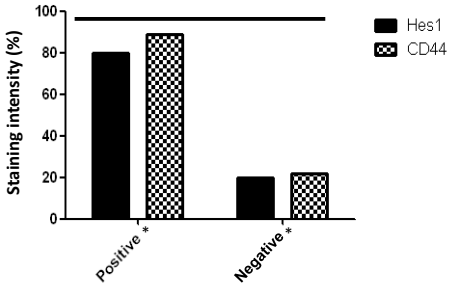
Important to our study gastric cancer patients have shown high expression level of wnt5a/b and the expression levels were well correlated to poor survival of these patients (55, 104).Therefore in accordance with the above mentioned study, we also wanted to analyze the expression of the same markers (CD44, Hes1, wnt 5a/b) in fresh human gastric cancer tissues (Normal-N, Gastritis-G, Gastric cancer – GC) (figure 10C). Our western blot results clearly show the highest expression of specifically CD44, Hes1 and also wnt 5a/b in gastric cancer tissues as compared to normal, gastritis human samples. The lowest expression of the targets were observed in normal tissues. We further wanted to analyze the coexpression of Hes1 and CD44 in the same population of $CD44^+$ MKN45 cells. In order to achieve $CD44^+$ MKN45 cells, these cells were subjected to sorting using FACS for CD44. FACS analysis of the CD44 sorted MKN45 cells also showed a considerably high expression of Hes1 (79.2%) in comparison to the $CD44^-$ population of MKN45 cells (22%) (Figure 10 D). The kaplan-meier survival analysis of 269 patients showed a strong correlation between CD44 and Hes1 double positivity with overall survival. Patients with both CD44 and Hes1 double positivity showed lower survival as compared to patients with Hes1 and CD44 double negetive (P=0.004) (Figure10 F).

Cell line	MKN45	KATO III	2313287	SNU	NCI-N87
CD44$^+$ (%)	65	18.4	8.7	4.5	4.5









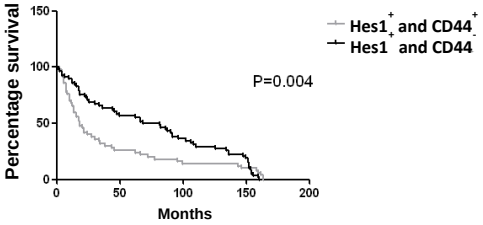


Figure 10: CD44 and Hes1 expression in gastric cancer cell lines and human tissues.

A.Table showing the relative percentage of expression of CD44 from a panel of 5 different human gastric cancer cell lines. Western blot analysis of the targets (CD44, Hes1, wnt 5a/b) in **B**.human gastric cell lines and **C**. human gastric tissues (N-Normal, G-Gastritis, GC-Gastric cancer). Beta-actin was used as a loading control. **D.** FACS analysis of CD44$^+$ and CD44$^-$ MKN45 cells showing a significant increase in Hes1 expression in CD44$^+$ population compared to the CD44$^-$. **E.** Bar graph representing the staining intensity of CD44 and HES1 in 269 human gastric cancer analysed tissues. (*GC tumour issues showing both moderate and strong staining for HES1 or CD44 were considered to be positive whereas tumour tissues showing both weak and negative staining were considered to be negative) **F.** Kaplan-Meier overall survival curve for GC patients categorised by Hes1 and CD44 expression showing a significant difference. (**P<0.005)

We also performed immunohistochemistry and immunofluorescence in human gastric cancer tissues derived from 269 patients. 86% of the gastric cancer patient tissues showed a considerably high expression of both CD44 and Hes1 (Figure 11 A, B and C). Importantly the CD44 positivity was mainly confined to the cell membrane and the cytoplasm (cytoplasmic staining) whereas positive staining for Hes1 was mainly

confined to the nucleus (nuclear staining) in the same gastric cancer tissues. The staining intensity was determined as explained in material and methods section. Notably, specifically in immnunofluorescence staining of human gastric cancer tissues we not only observed positivity for CD44 and Hes1in the gastric tissues but also, a strong coexpression \ colocalisation of CD44 and Hes1 (Figure 11B, C and D).

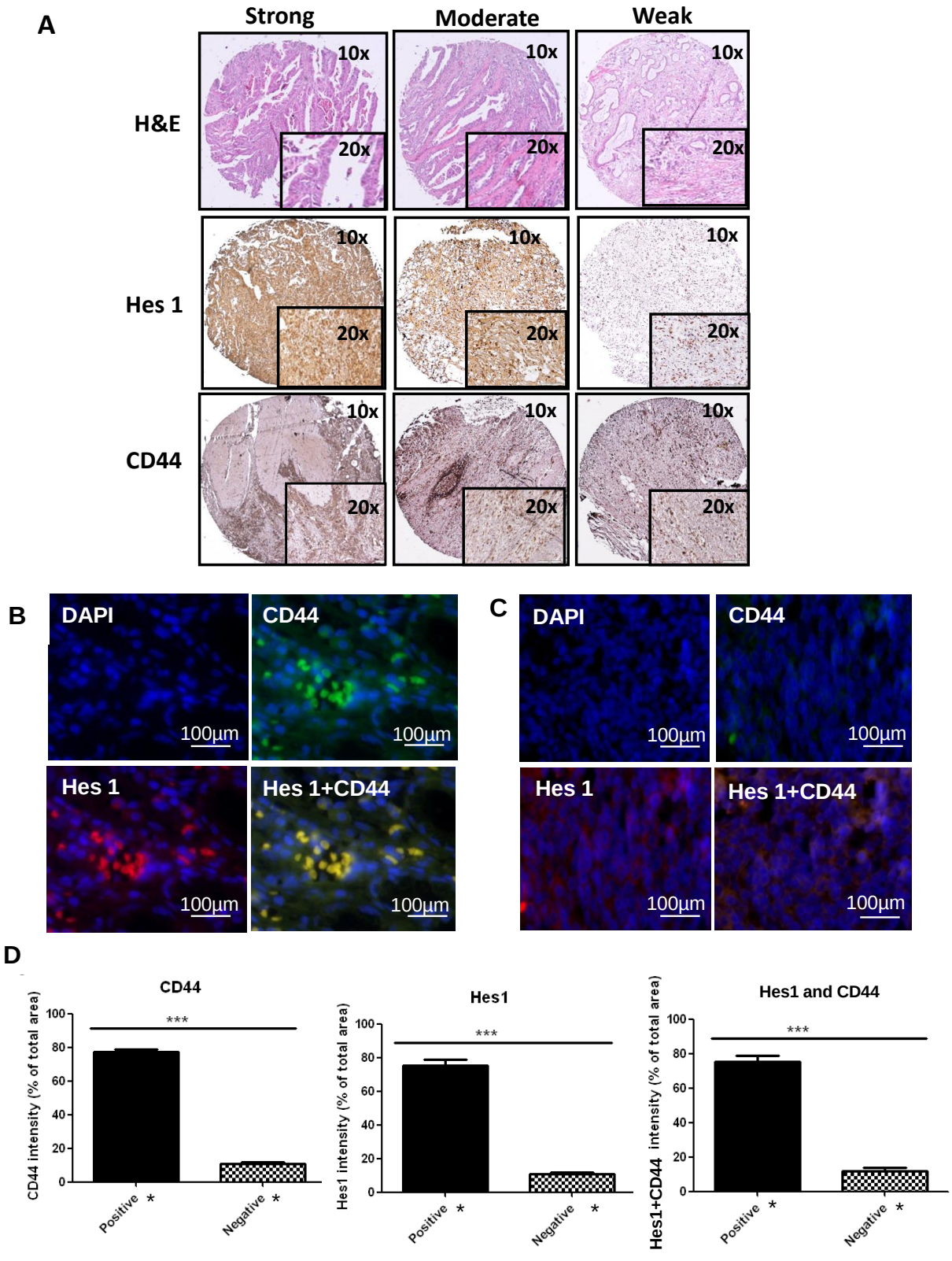


Figure11: Expression of CD44 and Hes1 in human gastric cancer tissues.

A.Immunohistochemical staining images of gastric cancer tissues showing the coexpression of CD44 and Hes1 with different staining intensities (strong, moderate, weak).Representative

pictures were taken using light microscope under (10X and 20X magnification. Immunofluorescence images of human GC tissues for CD44 (green), Hes1 (red) and 4,6-diamidino-2-phenylindole (DAPI) (blue) and merged (yellow). Representative images for **B.** positive staining **C**. Negative staining for CD44 and Hes1. Photographs were taken using confocal microscopy. **D.** Bar graphs representing the quantification of Hes1, CD44, CD44 and Hes1 (positive vs. negative) of 269 patients. *GC tumour tissues showing both moderate and strong staining for Hes1 or CD44 was considered to be positive whereas tumour tissues showing both weak and negative staining were considered to be negative ***P<0.001.

Next we wanted to examine if CD44, Hes1 double postivity can have on overall prognostic outcome or influence on patient survival. Therefore, in order to verify this we did a survival analysis of the patients from which the tumour tissues were derived showing positivity for both the targets ($CD44^{+}Hes1^{+)}$ (Figure 12 D). Interestingly, the analysis showed significantly lower or impaired survival in patients with double positivity for CD44 and Hes1 (P=0.004). We also observed differences in overall survival in patients with either CD44 or Hes1 positivity (P=0.004). Hes1 positivity showed a higher impact on overall survival, compared to CD44 single positive group of patients. Thus, the overall survival analysis shows that eventhough Hes1 single positive group of patients show lower survival than CD44 single positivity but CD44 and Hes1 double positive group of patients had the highest impact on overall survival being the lowest with a median survial of 35 to 40 months. A gender and age based study of the patients was also performed in order to confirm that these two factors might have an influence on the double positivity (CD44 and Hes1) in the patients. We did observe a higher expression of Hes1 and CD44 double positivity in older group of gastric cancer patients with an age reference range of 60 to 70 years. Unfortunately, no such difference or impact on incidence of double positivity was observed depending on the gender (male vs. female) (Figure 12 A and B).

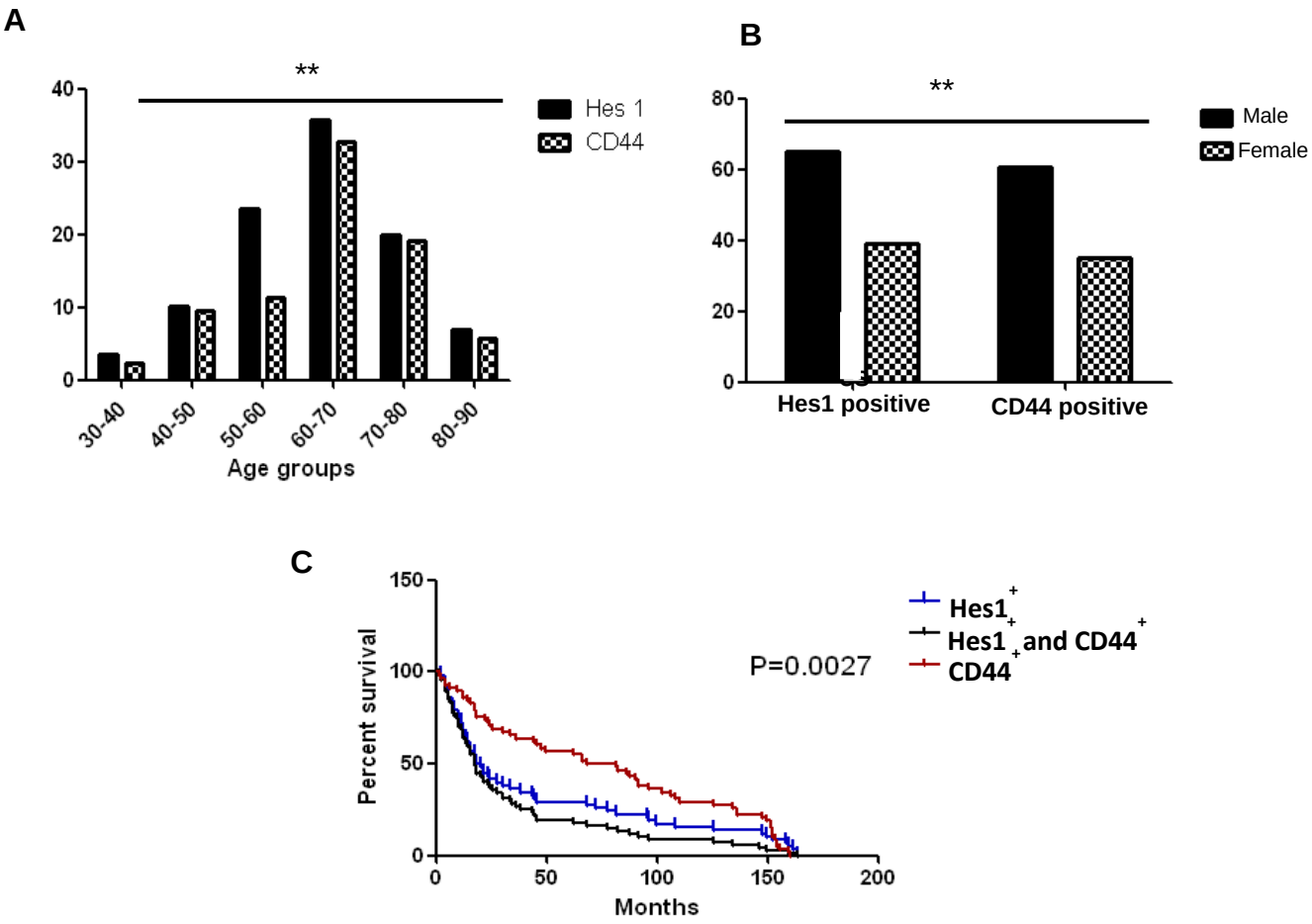


Figure 12: Age and Gender based incidence of CD44 and Hes1 positivity in human gastric tissues and its impact on overall survival.

Bar graph representing the staining intensities for CD44 and Hes1 according to **A**. Age and **B**. Gender (male vs. female) in a panel of 269 human GC tissues. * GC tumour tissues showing both moderate and strong staining for Hes1 and CD44 were considered to be positive whereas tumour tissues showing both weak and negative staining were considered to be negative **C**. Kaplan-Meier survival analysis of GC patients categorised by Hes1, CD44, Hes1 and CD44 expression ($P<0.005$).

Therefore, all together our results show that both human gastric cancer cell lines and tissues have a relatively considerable higher amount of double positivity and colocalisation for both CD44 and Hes1. CD44 and Hes1 double positivity can also act as a prognostic marker for gastric cancer patients as a strong correlation was observed in patients with double positivty ($CD44^{+}Hes1$) to the overall impaired survival. Important for our studies the gastric cancer cell line MKN45 showed positivity for CSC marker CD44 and also showed activation of both the pathways Notch (Hes1

positive) , wnt-beta-catenin pathways (wnt5a/b positive) known to be associated with CSC maintenance. Therefore, targeting Notch and wnt-beta-catenin pathways via CD44 could be of therapeutic interest in gastric cancer.

4.2GSI IX acts an effective anti proliferative, anti-migratory and anti-invasive agent and also induces considerable apoptosis in CD44$^+$ MKN45 cells.

To target CD44 and also to observe th effect of CD44 inhibition of MKN45 human gastric cancer cell line we used GSI IX inhibitor. Since, GSI IX is known to be associated with inhibition of Notch pathway, we tried to analyse if GSI IX treatment could also influence CD44 expression (CD44 being a CSC marker is also associated with the activation of Notch pathway, important for maintaining the self renewal of CSC population). Thus, we first tried to test the overall impact on CD44 inhibition using GSI IX on three main aspects associated with cancer growth: proliferation, invasion, migration ability of CD44$^+$ MKN45 cells.

Based on our previous screening we selected MKN45 as the candidate cell line for our further *in vitro* experiments as it showed significant activation of all the three targets (CD44, Hes1 and wnt5a/b). Uncontrolled cell growth or proliferation is one of the key mechanisms of cancer cells leading to cancer progression. Therefore, in order to inhibit proliferation of MKN45 gastric cancer cells we used GSI IX on CD44$^+$MKN45 cells. From our previous publication Palagani et al., 2012 (74) we chosethree specific dosages (2.5uM,5uM and 15uM) of GSI IX for treating MKN45 cells. Therefore, to verify if these three dosages can inhibit proliferation of CD44$^+$ MKN45 cells and that GSI IX specifically targets CD44 as a mode of action for inhibiting proliferation of CD44$^+$MKN45 cells we performed a time course cell proliferation assay. CD44 positive sorted population of MKN45 cells were treated with GSI (2.5uM,5uM and 15uM) for 24,48,72 and 96 hours using WST-1 reagent. The graph showed a significant inhibition of CD44$^+$ MKN45 cells in a dose and time dependent manner (Figure 13 A, B). The highest inhibition of CD44$^+$ MKN45 cells was observed at 15uM of GSI IX at 96 hours of treatment. Importantly, GSI IX effectively inhibited proliferation of only CD44$^+$ population of MKN45 cells as CD44$^-$ cells. In order to ensure that GSI IX is an effective drug for gastric cancer treatment we compared the anti-proliferative effect of GSI IX to 5FU. 5FU is a standardised

chemotherapeutic drug widely used for treating gastric cancer patients worldwide. Proliferation assay was performed using both $CD44^+$ and $CD44^-$ MKN45 cells and were treated with 5 FU in the highest dosage used for GSI IX treatment (15uM). The highest dosage of GSI IX (15uM) was also used for 5FU in order to compare the effectiveness of the drug at the highest possible dosage used for the study. A similar time course proliferation curve was obtained. The result clearly showed that 5FU treatment was more effective in inhibiting the growth of $CD44^-$ population of MKN45 cells as compared to the $CD44^+$ (Figure 13 C and D). Thus, this result clearly shows that GSI IX treatment has strong target specific anti-proliferative effect on $CD44^+$ MKN45 as compared to the standardised treatment option of 5FU. Morover, GSI IX effectively inhibits the growth of $CD44^+$ gastric CSC in contrast to 5FU, thus reducing the chances of tumour relapse associated with CSC.

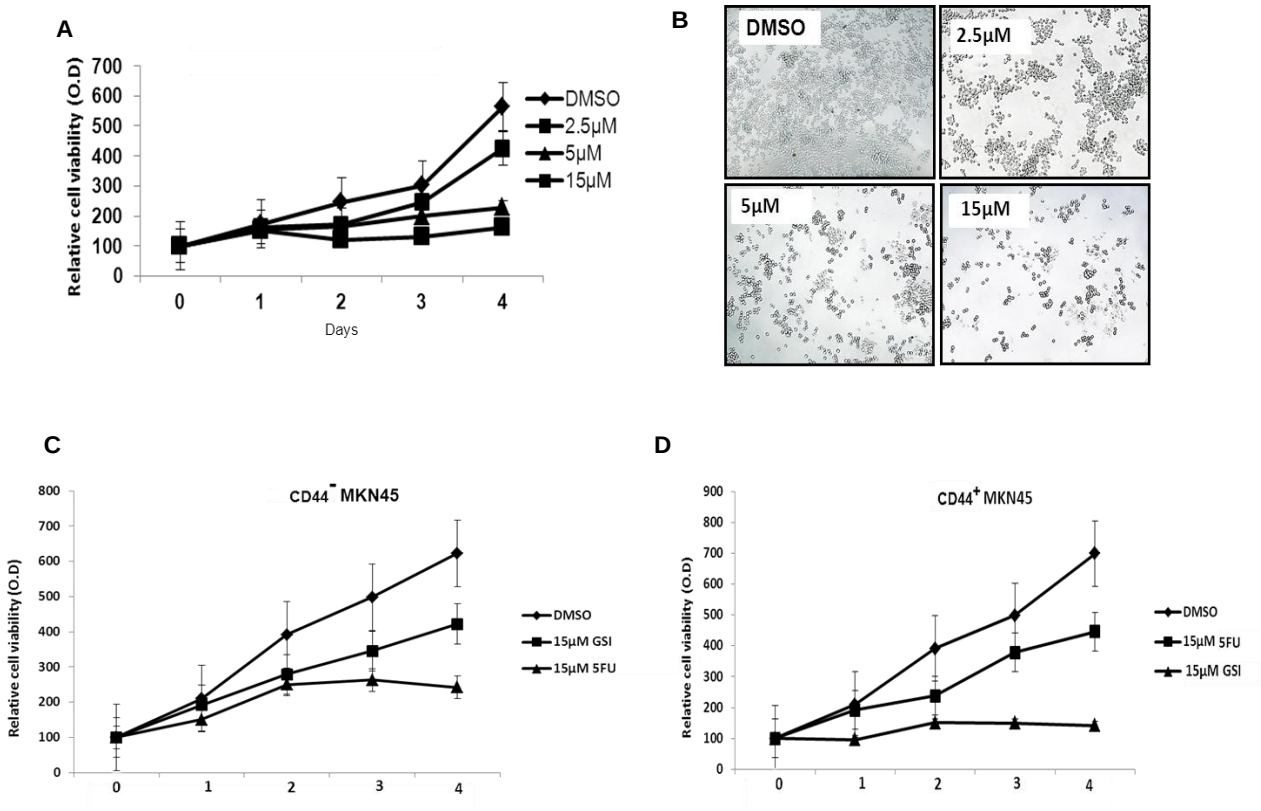


Figure 13: GSI IX effectively inhibits proliferation of $CD44^+$ MKN45 cells specifically via targeting $CD44^+$ CSC.

A.Line graph representing the cell proliferation of $CD44^+$ MKN45 cells treated with GSI (2.5, 5 and 15uM) or DMSO for 4 days. **B**. Corresponding light microscope images of cell proliferation assay on day 4 showing the anti-proliferative effect of GSI IX on $CD44^+$ CSCs.

Graph showing the comparison of target specific anti-proliferative effect of 15uM 5FU and 15uM GSI on **C**. $CD44^-$ population **D.** $CD44^+$ population of MKN45 cells. 5FU showed significant anti-proliferative effect only on $CD44^-$ population as compared to $CD44^+$ MKN45 cells. GSI IX effectively inhibits proliferation of both $CD44^+$ and $CD44^-$ MKN 45 cells.

Target specific action of GSI IX on $CD44^+$ was strongly observed on immunofluorescence staining of $CD44^+$ MKN45 cells for DAPI, CD44 and Hes1 treated with DMSO or GSI (2.5uM, 5uM and 15uM) (Figure 14A). Immunofluorescence staining clearly showed an effective inhibition or lower expression of CD44 and Hes1 single or colocalised expression in $CD44^+$ MKN45 cells by GSI IX.The strongest inhibition was observed under 15uM of GSI IX treatment as compared to the control. Thisresult revealed that GSI IX inhibits proliferation of MKN45 cells via specifically targeting $CD44^+$ CSC present in MKN45 cells.GSI IX mediated downregulation of CD44 also showed effective inhibition of Hes1 (Notch downstream).

A

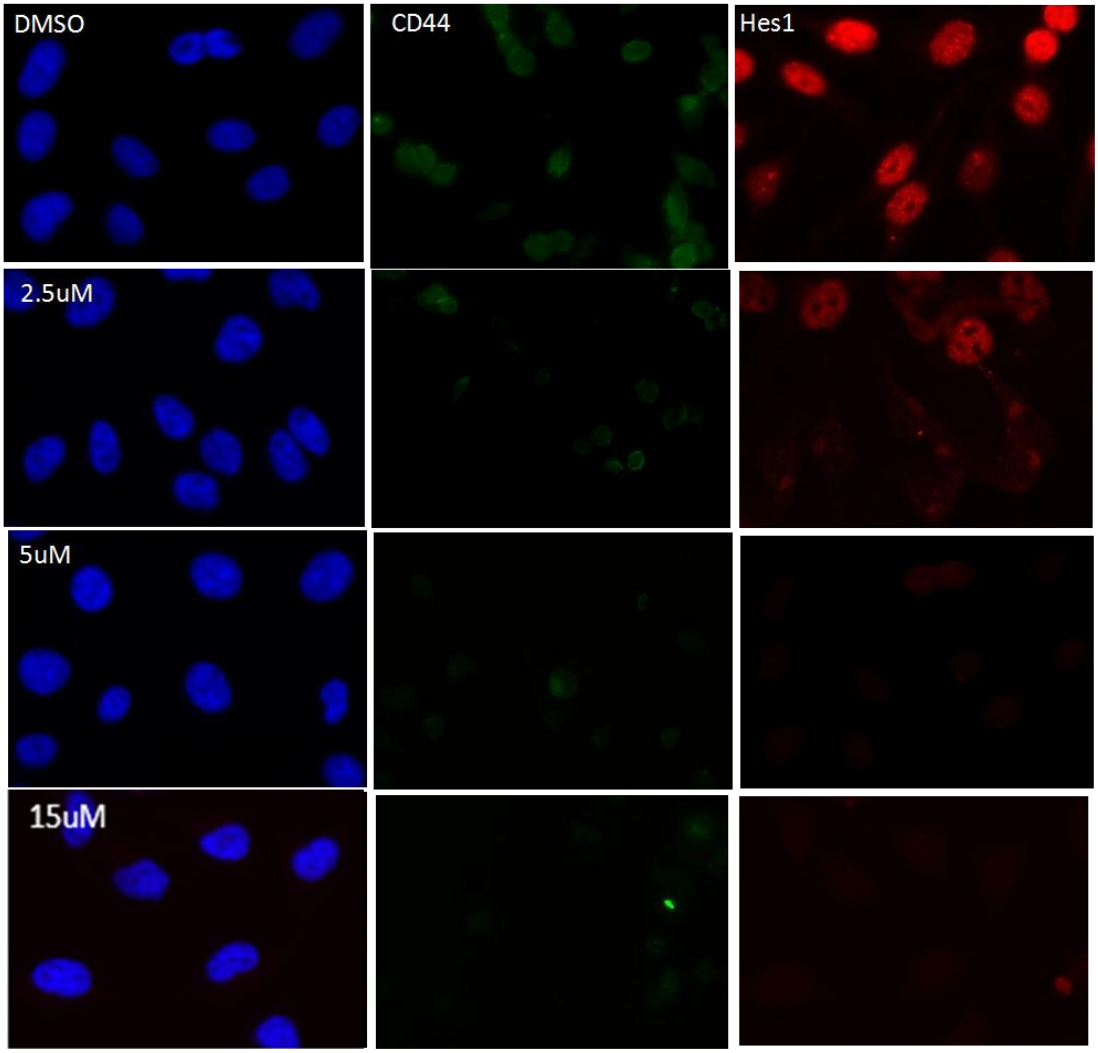


Figure 14: GSI IX effectively inhibits CD44 and Hes1 expression in the same population of CD44$^+$ MKN45 cells.

A.Immunofluorescence images for staining of 4,6-diamidino-2-phenylindole (DAPI, blue), CD44 (green), Hes1 (red) in CD44$^+$ MKN45 cells treated with DMSO or GSI (2.5, 5 and 15uM). Photographs were taken using confocal microscopy.

Invasion and migration are the two main important, higly integrated and multi-step processes involved in cancer progression or metastasis. An effective therapeutic approach is considered to be optimal if the tested drug shows potential as an anti-proliferative, anti-invasive and anti-migratory agent on cancer cells. Therefore, our next step was to determine if GSI IX treatment caneffectively inhibit invasion and migration of CD44$^+$ MKN45 cells. Therefore, we first performed migration or wound healing assay. The CD44$^+$ MKN45 cellswere treated with GSI (2.5uM, 5uM and 15uM) or DMSO and was subjected to wound healing analysis. Significant (P=0.001) inhibition of migration was observed under GSI IX treament in CD44$^+$ gastric cancer

cells compared to the control Migration is usually analysed by measuring the relative closure of the wound in $CD44^+$ MKN45 cells under GSI IX treatment in comparison to the control. The highest anti-migratory effect was observed under 15uM of GSI IX treatment in comparison to the DMSO control. (Figure 15 A).

Next we wanted to examine invasion using matrigel coated invasion chambers after GSI IX treatment of $CD44^+$ MKN45 cells. GSI IX treatment effectively inhibited the invasion of $CD44^+$ MKN45 cells in concentration dependent manner ($P<0.001$) (Figure 15 B). The most effective inhibition was observed under 15uM GSI IX treatment. Thus, our results show that GSI IX treatment effectively inhibits migration and invasion of $CD44^+$gastric cancer cells.

GSI IX treament effectively inhibited proliferation and also induced considerable cell death. Therefore, we wanted to analyse the exact mechanism by which GSI IX Notch inhibitor is inducing cell death on the $CD44^+$ MKN45 cells. Apoptosis also known as programmed cell death is a systemic process in which cells commit suicide by activating an intracellular porgramme. Apoptosis is the one of the major key mechanisms or process by which cells undergo death by activating an intracellular proteolytic cascade (72). Importantly, apoptosis is often considered to be one of the main mechanisms involved incancer cell death. So, we analysed apoptosis using Annexin V/PI staining and measured the amount of apoptotic cells using FACS. (Annexin V is usually used in conjunction with another vital dye PI. Viable cells with intact cell membrane exclude PI therefore are PI negative whereas cells with distorted cell membranes are permeable to PI therefore, dead cells are PI positive. Early apoptotic cells are Annexin V+ but PI- whereas late apoptotic or nearly dead cells are both Annexin V+ and PI+). We noted that GSI IX treatment showed significantly ($P>0001$) high induction of apoptosis as compared to the DMSO group (Figure 16 A). The highest induction of apoptosis of $CD44^+$ MKN45 cells was observed at 96 hours under 15uM of GSI IX treatment. Thus, the results highlightedapoptosis as the main mechanism by which GSI IX induces cell death in $CD44^+$ MKN45 cells.

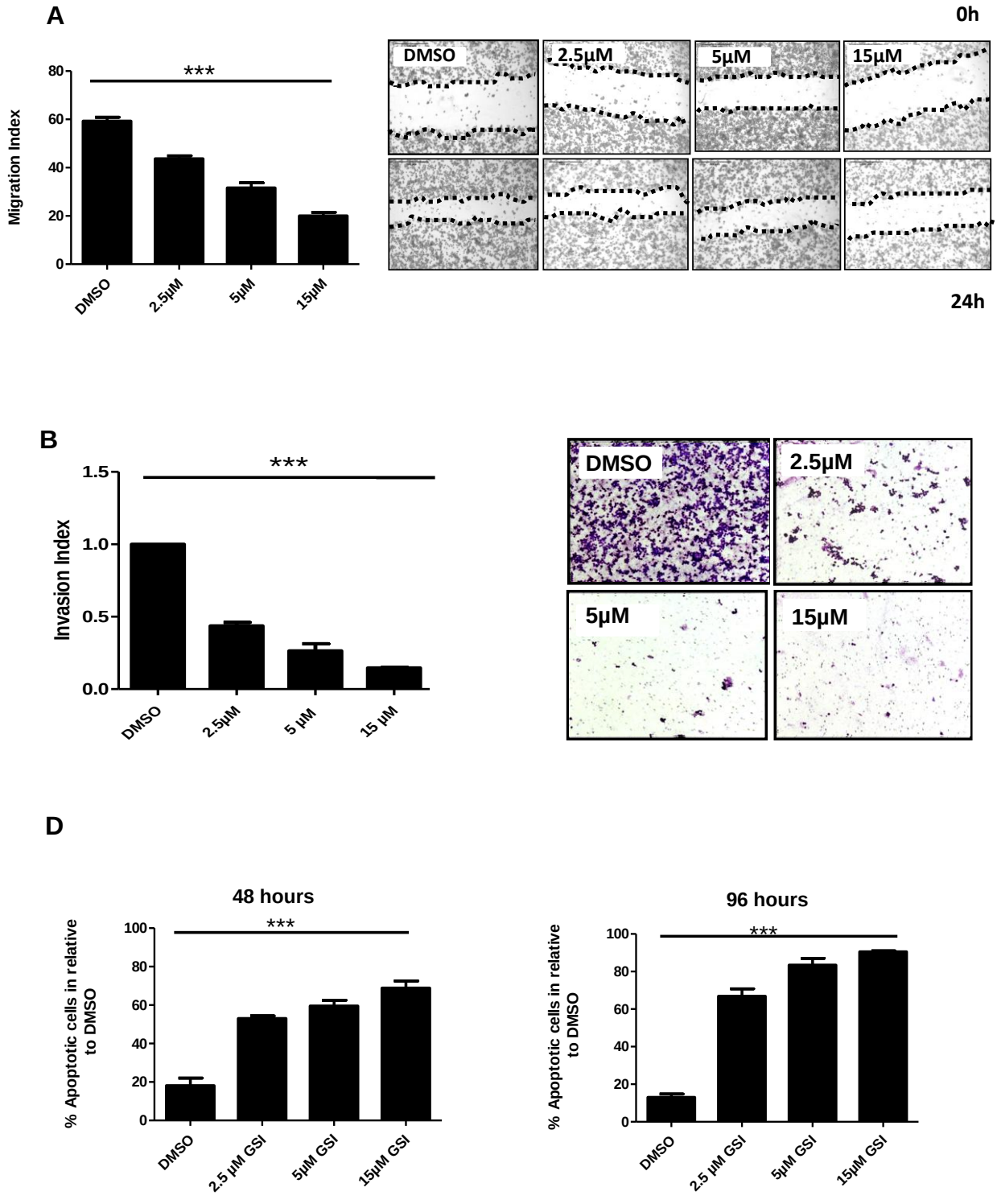
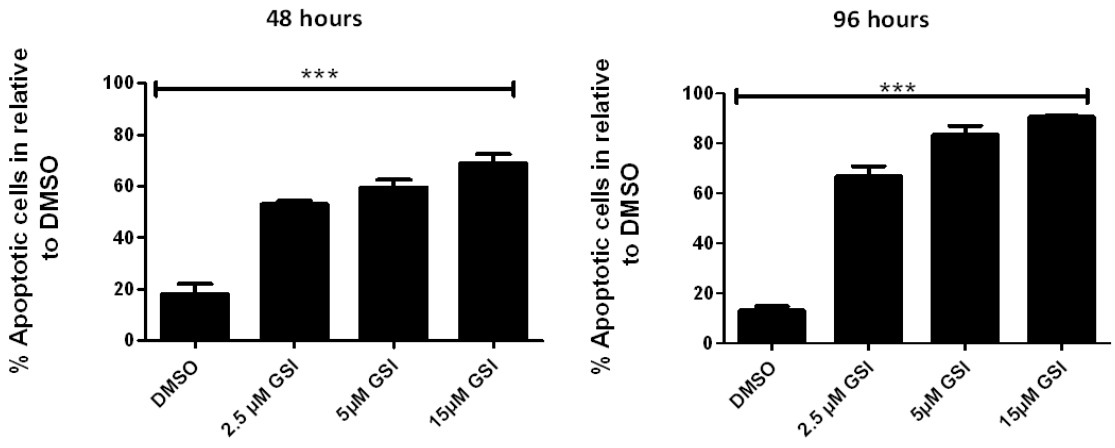


Figure 15: GSI effectively inhibits migration, invasion and induces apoptosis in CD44$^+$ MKN45 cells.**A.**Bar graph representing the Migration index of CD44$^+$ CSCs after DMSO or GSI (2.5uM, 5uM and 15uM) treatment. Photographs to analyse the relative wound

healingwere taken at 0hour and 24 hours from the time the scratch was made (20X magnification). **B**. Bar graph representing the invasion index of $CD44^+$ CSCs after DMSO or GSI (2.5, 5 and 15uM) treatment. Light microscope pictures (20X magnification) were taken and invasion index was calculated

A.



B.

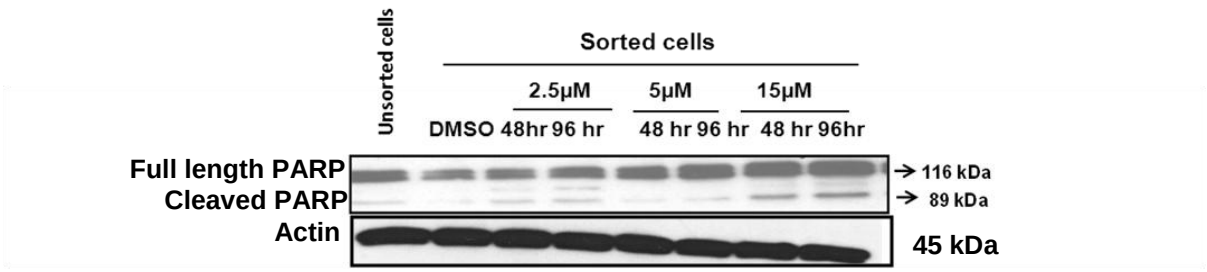


Figure 16: GSI effectively inhibitsinduces apoptosis in $CD44^+$ MKN45 cells.

A. Apoptosis was quantified using Annexin V/propidium iodide (PI) staining using flow cytometry to $CD44^+$ MKN45 cells treated with DMSO or GSI (2.5uM, 5uM and 15 uM) for 48 and 96 hours***($P<0.001$).**B**. Western blot showing the clevage of PARP under GSI treatment (2.5uM, 5uM and 15 uM). Actin was used as a loading control.

Recent studies showed that poly(ADP-ribose) polymerase (PARP) is required for inducing apoptosis in several cells. Cleavage of PARP catalysed by caspase-3 which leads to the depletion of NAD (a PARP substrate) and ATP which are important for

later events of apoptosis. The importance of cleavage of PARP in apoptosis initiation was first investigated and proved in human osteosarcoma cells (73). Thus we also wanted to check for cleavage of PARP as a marker for apoptosis in GSI IX treated $CD44^+$ MKN45 cells. As Annexin V/PI staining showed a considerable induction of apoptosis therefore, we thought checking of the status of PARP by western blot in $CD44^+$ MKN45 cells treated withDMSO or GSI IX (2.5 uM, 5 uM and 15uM) could be of interest. Western blot analysis showed a higher expression in clevage of PARP under GSI treatment specifically under 15uM of GSI IX in $CD44^+$ MKN 45 cells at 96 hours (Figure 16 B). As expected the western blot results of PARP as a marker for apoptosis showed us a similar result in accordance with our previous result of apoptosis measurement by FACS. These results thus reconfirmed apoptosis as one of the key mechanisms involved in induction of cell death by GSI IX treatment in $CD44^+$ gastric CSCs.

Therefore, GSI IX not only targets $CD44^+$ population of MKN45 cells resulting in inhibition of invasion, migration but also induces high amout of apoptosis.

4.3 GSI IX treatment effectively impairs tumoursphere formation of $CD44^+$ MKN45 cells.

CSCs harbor the potential to form non-adherent spheres or tumourspheres under low attachment conditions. This phenomenon of CSCs is also known as the de novo tumour formation. Therefore, it would be of interest to see if GSI IX treament can influence tumoursphere formation of $CD44^+$ MKN45 cells. GSI IX (2.5uM, 5uM and 15uM) treatment showed effective inhibition of tumour sphere formation in $CD44^+$ MKN45 cells as compared to the DMSO control ($P>0.001$). Notably, the size of the spheres formed by the control cells were relatively larger than that formed by the $CD44^+$ MKN45 cells under GSI IX treatment (Figure 17A, B and C). The size of the spheres reflect that GSI IX treatment inhibits the tumour sphere forming potential of $CD44^+$ gastric CSCs which cause lesser accumulation of cells to form tumourspheres which are smaller in size compared to the untreated cells.

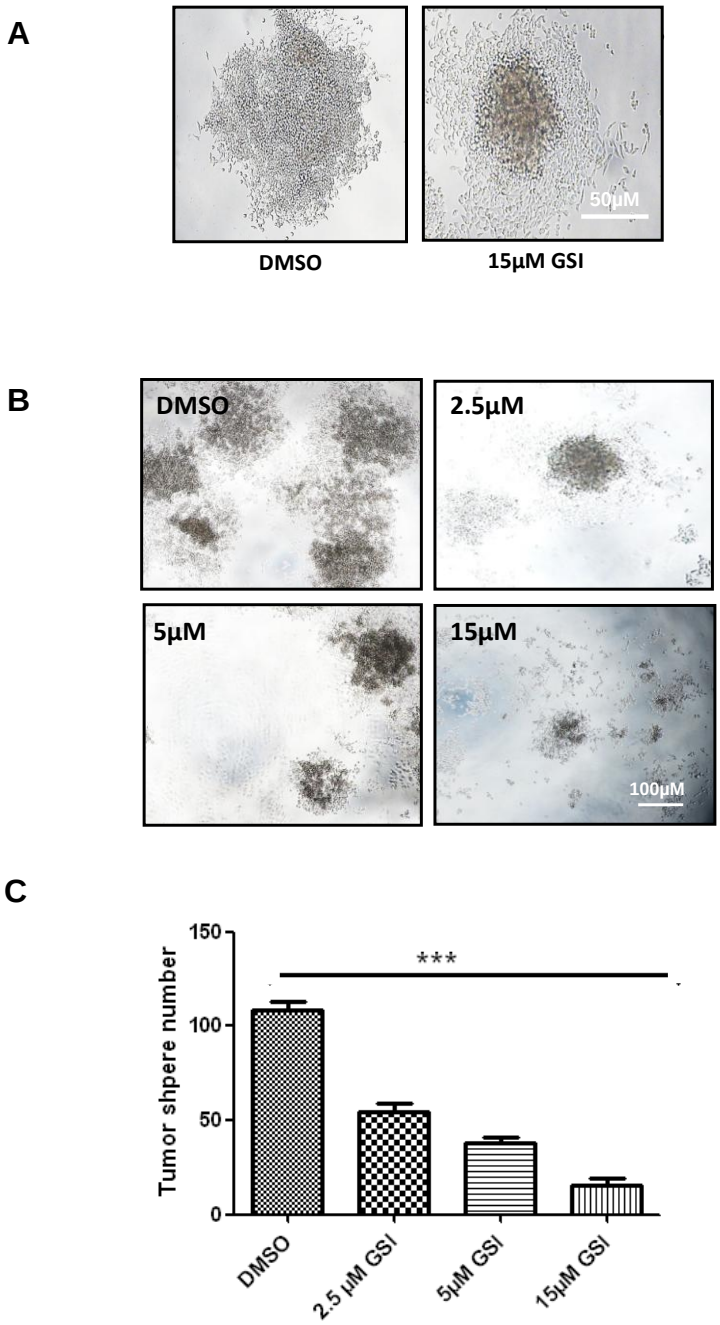


Figure 17: GSI inhibits tumoursphere formation in CD44$^+$ MKN45 cells.

A.Representative images of tumourspheres formed by control (DMSO) and 15uM GSI treated CD44$^+$MKN45 cells. Light microscope pictures of tumourspheres formed after GSI treatment in comparison to the DMSO control at 96 hours**B**. CD44$^+$MKN45 cells were treated with DMSO or GSI (2.5uM, 5uM and 15 uM) for 96 hours. **C**. Bar graph showing the quantification of the tumourspheres formed by CD44$^+$ MKN45 cells after DMSO or GSI treatment (*** P<0.001)

4.4GSI IX effectively blocks CD44 mediated adhesion in $CD44^+$ MKN45 cells.

CD44 belongs to the family of adhesion molecules involved in the cell to cell and cell matrix interaction. CD44 adhesion molecule under certain conditions also initiates metastatic spread of tumour cells (49). Adhesion could also be one of the probable mechanism achieved by CD44 for tumour spread. Therefore, we tested the effect of GSI IX treatment on the adhesive capacity of CD44. Adhesion assay results showed an effective increase in suspension cell population under GSI IX treatment (2.5uM, 5uM and 15uM) (20-25%; 55-60%; 80-85% respectively) in comparison to the DMSO treated control cells (40-45%). Hyaluronic acid (HA) is an important ligand for CD44 binding. Thus, for adhesion assay HA coated plates were used and the population of suspension and adherent cells in $CD44^+$ MKN45 cells was measured under different conditions. $CD44^+$ MKN45 cells showed decrease in the population of adherent cells resulting in increase in suspension cells specifically under 15uM of GSI IX treatment as compared to control. This shows that GSI IX treatment effectively targets and inhibits CD44 expression resulting in inhibition of the role of CD44 as a adhesion molecule in $CD44^+$ gastric cancer cells. Importantly, $CD44^+$ MKN45 cells showed an increase in adhesion cell population due to increased affinity of $CD44^+$ cells to bind tothe only ligand HA. Therefore, GSI IX treatment effectively targets and inhibits CD44 adhesion molecule associated with gastric cancer progression (Figure 18 A,B and C).

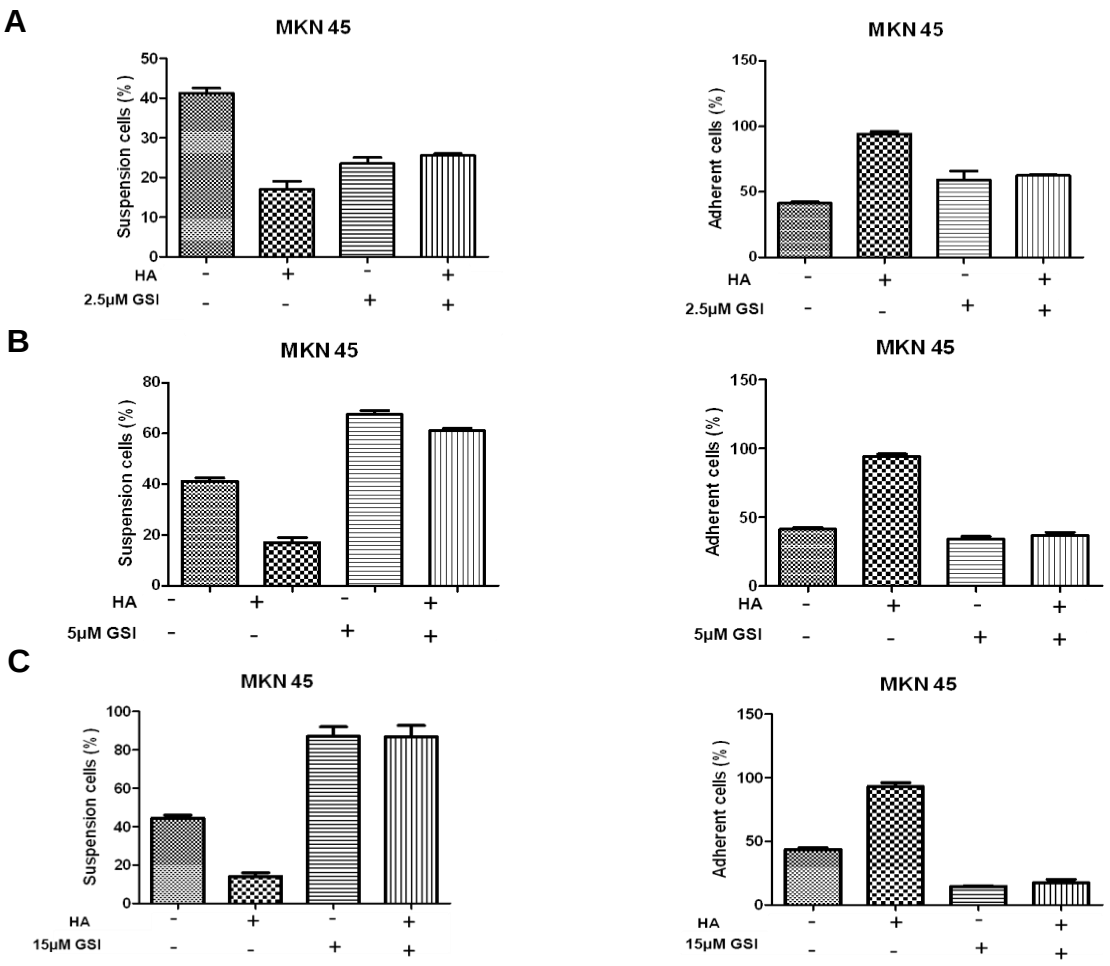


Figure 18: GSI effectively inhibits adhesion of CD44$^+$ MKN45 cells

Bar graphs representing the amount of adherent and suspension cells present in CD44$^+$ MKN45 cells after **A**. 2.5uM GSI, **B**. 5uM GSI and **C**.15uM GSI treatment under with or without hyaluronic acid (HA, the ligand for CD44 binding) (*** P<0.005).

4.5 GSI IX effectively inhibits Notch1 mediated concomitant activation of Notch and wnt-beta-catenin pathways in CD44$^+$ MKN45 cells.

Notch pathway activation involves activation of several Notch receptors like Notch 1, Notch 2 and Notch 3. Since our western blot and immunofluorescence results clearly show the activation of Hes1 (a downstream of Notch) in CD44$^+$ MKN45 cell line as well as in human gastric cancer tissues therefore, we wanted to see which Notch receptor is important to activate the Notch pathway in CD44$^+$ gastric CSCs. Mainly we were interested in identifying the downstream targets that might play a role in Notch mediated gastric cancer progression and impaired upon GSI IX treatment. For this the CD44$^+$MKN45 cells were treated with DMSO or GSI IX (2.5uM, 5uM and 15uM) and were subjected to western blot analysis. Interestingly, the western blot results showed a relative downregulation of specifically Notch1 after GSI IX treatment in a dose and time dependent manner whereas, Notch 2and Notch 3 remained relatively unchanged (Figure 19 A). Moreover, the intracellular domain of Notch1 (NICD) also showed a relative downregulation compared to the DMSO control under GSI IX treatment. c-Myc is an oncogene that is overexpressed in majority of human cancer and also contributes to about 40% of all tumours. Several studies specifically the study by Herranz Det al.,2014 (69) showed that in certain malignancies like T cell lymphoblastic leukeamia Notch1 causes a c-Myc mediated feed forward loop which results in c-Myc dependent cellular growth and metabolism in cancer. Several reports also showc-Myc as a direct downstream of Notch1 in cancer. Since Notch 1 was observed to be downregulated by GSI IX in CD44$^+$ gastric cancer MKN45 cells,we also checked for c-Myc status before and after GSI IX treatment in these cells. By western blot analysis we observed a relative downregulation or inhibition of c-Myc after GSI IX treatment. The highest inhibition was observed in 15uM of GSI IX treatment at 96 hours. Hence, this shows that GSI IX might inhibit Notch signaling in gastric cancer CSCs via inhibition of Notch1 and its downstream c-Myc in CD44$^+$ MKN45 cells. Therefore, Notch1 and c-Myc might mediate a feedback loop not only in gastric cancer progression but also in GSI IX mediated treatment in CSCs (69). Notch1 and c-Myc also has several common downstream effector proteins like Ras-related C3 botulinum toxin substrate 1 (Rac-1) associated with cell motility during carcinogenesis, Akt (Serine/threonine kinase also known as protein kinase B) responsible for cell survival in cancer, extra

cellular signal regulated kinase regulates (ERK) cell proliferation, signal transducer and activator of transcription 3 (Stat3) is known to play as an oncogene and promote cancer via activation of several cancer associated pathways.Therefore,using western blot we wanted to confirm the inhibition of these common downstream effector proteins in GSI IX treated $CD44^+$ MKN45 cells (Figure 19 B). Thus, the phospho-form of these downstreams which are known to play important role in cell regulatory pathways (Rac1, AKT, Erk, STAT3) were effectively downregulated under GSI IX treatment whereas, the full forms of these effector proteins remained unchanged after treatment. Therefore, GSI IX treatment inhibits Notch1 and c-Myc which results in complete inhibition of common important downstreams like Erk, Akt etc in GSI IX treatment in $CD44^+$ gastric cancer cells.

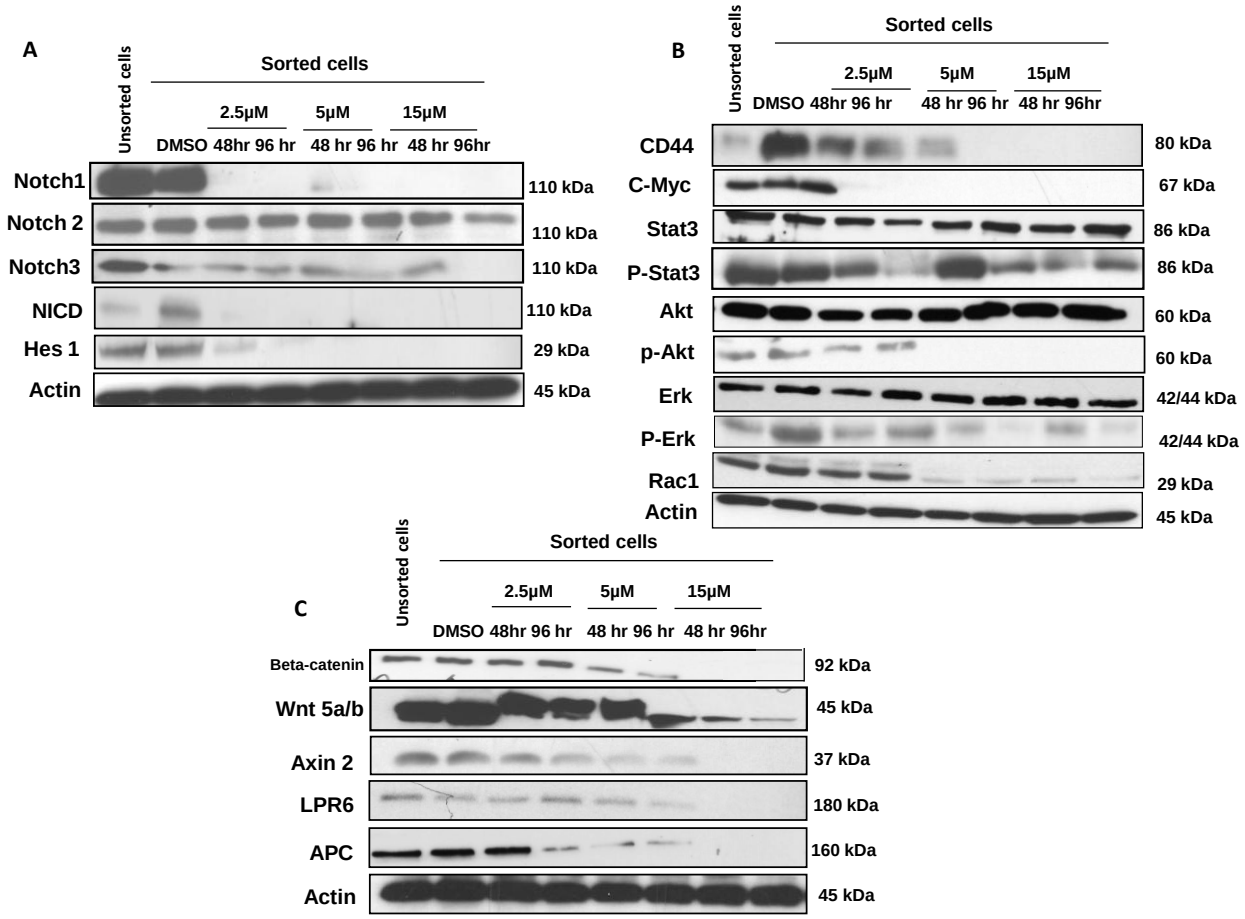


Figure 19: GSI effectively inhibits Notch and wnt-beta-catenin crosstalk in $CD44^+$ MKN45 cells.

$CD44^+$ MKN45 cells were treated with DMSO or GSI (2.5uM, 5uM and 15uM) for 48 hours and 96 hours **A**. GSI treatment was evaluated on Notch signaling downstream effector proteins (Notch1, Notch 2, Notch3, NICD, Hes1) by western blotting.**B**.GSI effect on probable candidates involved in Notch and wnt-beta-catenin crosstalk in $CD44^+$ gastric cancer cells (CD44, c-Myc, Stat3, p-Stat3, Akt, p-Akt, Erk, p-Erk, Rac1) analysed by western blotting. **C**. Western blots showing the effect of GSI treatment on wnt-beta-catenin pathway downstreams in $CD44^+$ MKN45 cells (Beta-catenin, wnt 5a/b, Axin 2, LPR6, APC). Actin was used as a loading control.

Since Notch and wnt-beta-catenin pathways are the two main players involved in maintenance of CSCs, we next wanted to analyse whether wnt-betacatenin pathway can also be inhibited via GSI in $CD44^+$ MKN45 cells. Therefore, we checked for some common wnt-beta catenin pathway downstreams like Axin-related protein (Axin2) , APC (adenomatous polyposis of the colon), LPR6 after GSI IX treatment.
InWnt pathway the formation of a multi-protein complex mediated by Axin 2 consisting of APC, beta-catenin, glycogen synthase kinase 3-beta, LPR6 results in degradation of beta catenin. Thus, this complex is important for regulation of beta catenin in wnt pathway (76).In order to understand the status of wnt-beta-catenin pathway in $CD44^+$ MKN45 after GSI IX we checked the above mentioned wnt downstreams using western blot. Western blot analysisclearly showed a relative inhibition of the downstreams of wnt-beta-catenin pathway (Wnt 5a/b, beta-catenin, Axin2, APC, LPR6) under GSI IX treament in $CD44^+$ MKN45 cells (Figure 19C). Thus our data clearly shows that the co activation of both Notch and wnt beta-catenin pathway in $CD44^+$ gastric CSCs. Moreover, these results also proove that GSI IX treatment effectively inhibits the concomitantly active Notch and wnt-beta-catenin pathways in $CD44^+$ gastric CSCs via inhibition of common downstream effectors. Importantly, this concomitant activation of both the pathways in gastric CSCs might be mediated via Notch-1 and c-Myc (Notch -1 activates c-Myc which in turn activates a series of downstream effector proteins also common to wnt-beta catenin pathway).

4.6GSI IX partially influences EMT in $CD44^+$ MKN45 cells.

Epithelial Mesenchymal Transition (EMT) is a critical process during tumour progression and malignant transformation. During EMT epithelial cells acquire

mesenchymal phenotype thus endowing the cancer cells with invasive and metastatic potential. Many oncogene pathways including Notch and Wnt-beta-catenin induce EMT process.EMT is commonly characterised by the down regulation of epithelial marker E-Cadherin and upregulation of mesenchymal marker N-cadherin during cancer progression (70). We therefore, wanted to asses the impact of GSI IX treatment on EMT markers specifically E and N cadherin. Western blot analysis showed that GSI IX treatment partially influenced the EMT process as E-cadherin remained relatively unchanged whereas, N-cadherin was partially downregulated specifically under 15uM of GSI IX treatment in 96 hours (Figure 20 A). Therefore, GSI IX treatment has a partial impact on EMT process in $CD44^+$ gastric CSCs.

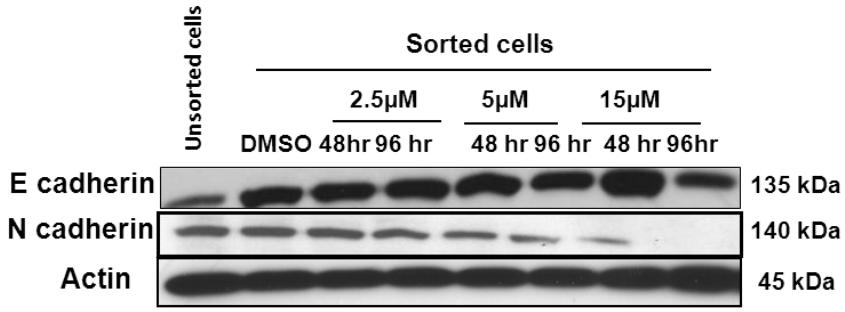


Figure 20: GSI IX partially influences EMT in $CD44^+$ MKN45 cells.
$CD44^+$ MKN45 cells were treated with DMSO or GSI (2.5uM, 5 uM and 15 uM) for 48 hours and 96 hours. Influence of GSI on EMT markers (E cadherin, Ncadherin) was analysed by western blotting on $CD44^+$ MKN4 cells. Actin was used as a loading control.

4.7 GSI IX blocks the growth of $CD44^+$ gastric cancer cells via inhibition of Notch1 mediated Notch and wnt-beta-catenin crosstalk in MKN 45 cells.

In order to understand the exact mechanistic approach of GSI IX in inhibition of $CD44^+$ CSCs, and moreover to understand the pivotal role of Notch and CD44 in gastric cancer progression we performed siRNA mediated silencing of both CD44 and Notch1. Western blot was used to analyse the effect of siRNA mediated silencing of Notch1 and CD44 on Notch, wnt-beta-catenin pathway and the common downstream effector proteins involved in mediating the crosstalk both with or without

15uM of GSI IX treatment. Firstly siRNA mediated silencing of CD44 led to the downregulation of Hes1, p-Erk, p-Akt, Rac1, p-Stat3, c-Myc,wnt 5a\b, CD44, Notch1 (Figure 21 A). These results were in accordance with the effect observed after GSI IX treatment. Notch1 siRNA treatment showed by immunoblotting similar down-regulation of CD44, Hes1, p-Erk, p-Akt, Rac1, p-Stat3, c-Myc, wnt 5a/b and beta catenin, these results were also in accordance to the effect observed under GSI IX treatment (Figure 21 B).Therefore, these results clearly show that GSI IX mediated approach for inhibition of gastric CSCs is mainly mediated by targeting the $CD44^+$ CSCs which in turn results in the Notch1 mediated inhibition of Notch wnt-beta-catenin crosstalk involved in the maintenance of gastric CSCs. Therefore, siRNA mediated silencing of CD44 and Notch1 not only demonstrated the status and importance of these two targets in mediating the Notch wnt-beta-catenin crosstalk in gastric CSCs but also highlighted the probable mechanistic approach exhibited by GSI IX in $CD44^+$ gastric cancer cells.

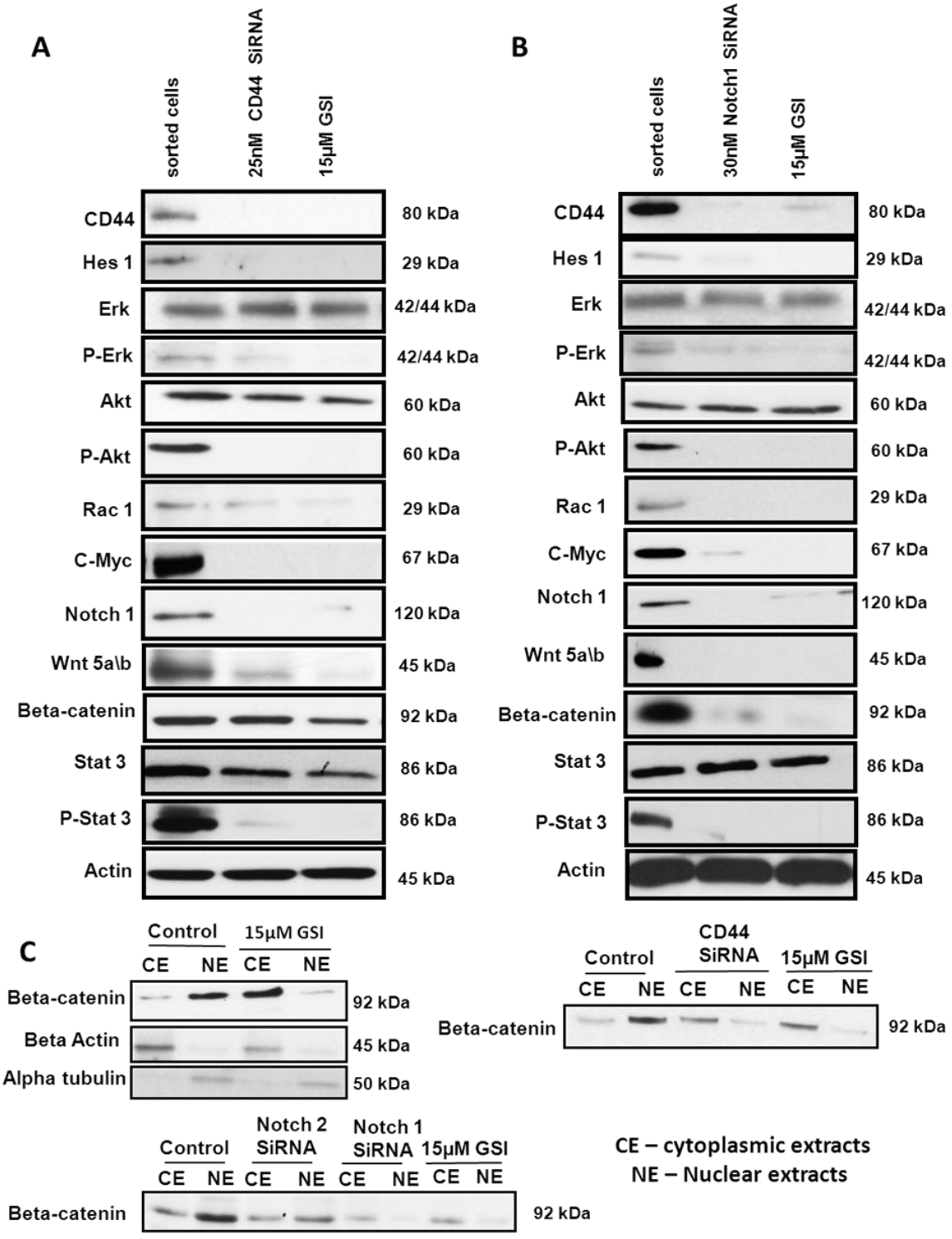


Figure 21: GSI inhibits Notch and wnt-beta catenin crosstalk in CD44^{+} MKN45 cells via Notch 1

siRNA mediated silencing of **A**. CD44 **B.** Notch1 to confirm the effect of GSI on Hes1, p-Erk, p-Akt, RAC1, Notch 1, c-Myc, Stat3, P- Stat3, wnt 5a/b, beta-catenin by immunoblot.

β-actin was used as a loading control. **C.** Complete inhibition of nuclear translocation of beta-catenin was observed after GSI treatment in $CD44^+$CSCs using western blot.Alpha-tubulin was used as loading control.

A major hallmark for the activation of the wnt-beta-catenin pathway is the accumulation of beta-catenin in the cytoplasm. wnt regulates beta catenin levels through various mechanisms as mentioned in Bryan T et al.,2009(71). One of the main mechanisms involved in the activation of this pathway is via the formation of APC-Axin2-GSK3B complex which can induce activation of various Wnt downstream targets via translocation of beta catenin to the nucleus. Importantly the status of beta catenin is important to determine the off set (accumulation in the cytoplasm) or on set (translocation to the nucleus) status of wnt-beta-catenin in $CD44^+$MKN45 cells. Therefore, to see the inhibition effect of GSI on wnt-beta-catenin pathway we performed western blot using the nuclear and cytoplasmic extracts of $CD44^+$ MKN45 treated with DMSO or GSI IX (2.5 uM, 5 uM and 15uM). Our western blot results clearly showed that 15uM GSI IX effectively inhibited translocation of beta catenin to the nucleus (Figure 21C). Thus, inactivation of wnt/beta-catenin pathway was observed after GSI IX treatment as, GSI effectively inhibited the nuclear transloction of beta cateninin $CD44^+$ MKN45 cells. The western blots clearly showed higher accumulation of beta-catenin in the cytoplasmic extract than the nucelar extract under GSI IX treatment in contrast to the controls. Therefore, GSI IX effectively inhibits not only the Notch pathway but also the wnt-beta-catenin pathway in $CD44^+$ gastric CSCs.

4.8GSI IX effectively inhibits tumour growth in $CD44^+$ MKN45 xenograft tumours.

Important to our study we next wanted to examine the *in vivo* effect of GSI IX on $CD44^+$ xenograft tumours. In order to achieve this each xenograft mice was injected with $CD44^+$ MKN45 cells on both flanks. Treatment protocol and method used to establish xenograft tumour in nude mice is explained in Materials and Methods section. Tumou r progression is one of the biggest challenge faced during cancer treatment therefore, inhibition of tumour growth is utmost importance in cancer therapy. In order to achieve this we treated $CD44^+$xenograft tumours with GSI IX

(10mg/kg four times per week) or placebo (PBS). In a total of 4 weeks of GSI IX treatment we observed a significant (P>0.001) difference in tumour size between the control (placebo treated) and GSI IX treated groups. GSI IX treatment effectively inhibited $CD44^+$ gastric tumour growth in xenografts (Figure 22 A,C and F). Histological analysis of resected xenograft tumours showed higher incidence of necrosis in the GSI IX treated group as compared to the placebo group (Figure 22B). Therefore, GSI IX treatment induces necrosisto inhibit tumour growth *in vivo* in $CD44^+$ MKN45 xenograft tumours.Moreover, GSI IX treated xenograft tumours showed significantly (P<0.005) lower tumour weight as compared to the control group (Figure 22 E). Importantly, GSI IX treated tumours showed a stable body weight during the treatment tenure as compared to the placebo treated xenograft group (Figure 22 D).

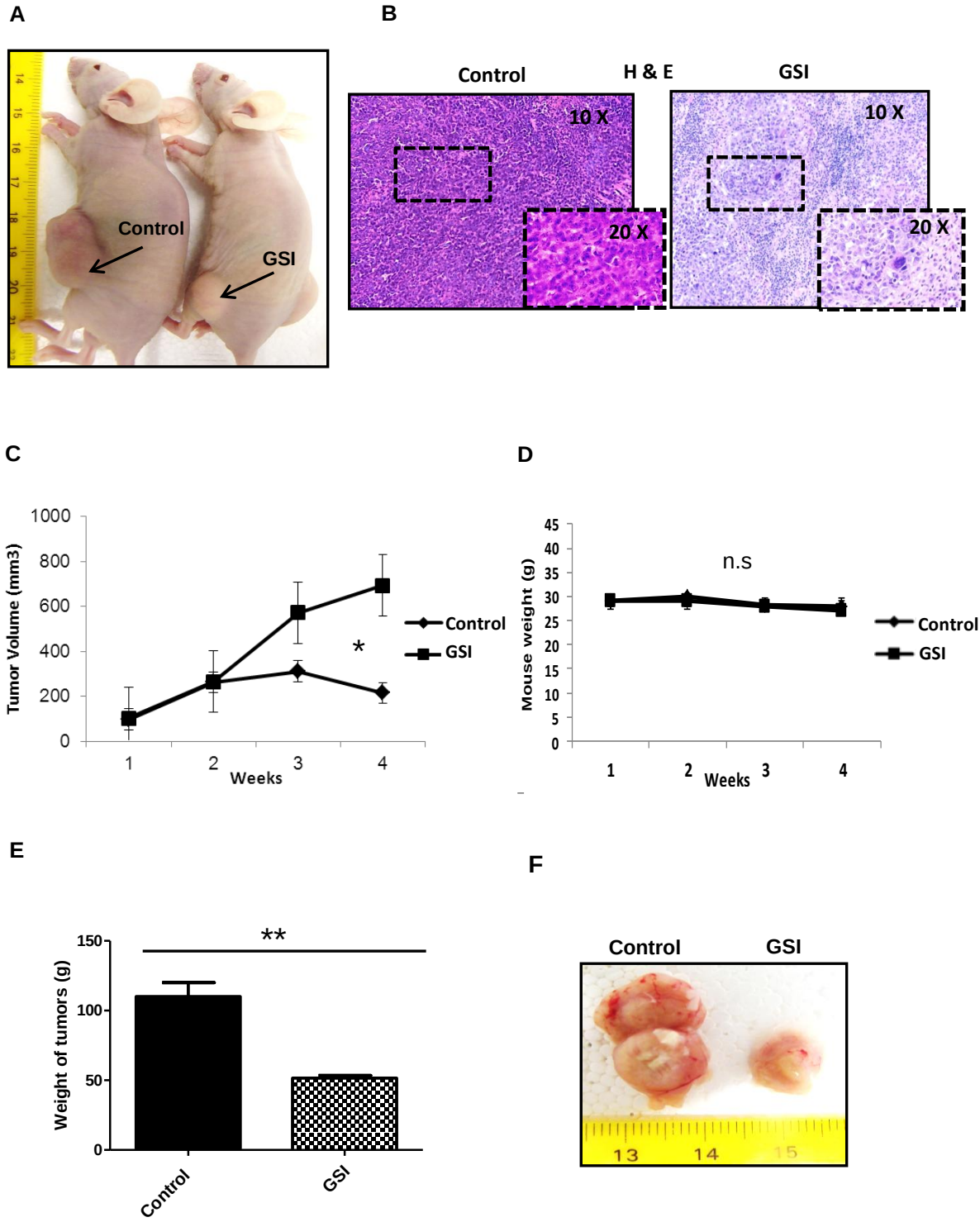


Figure 22: GSI inhibits CD44^{+} CSC tumour growth.

A.Pictures of mice taken at 4 weeks. The left mouse is treated with vehicle, the right mouse with GSI IX. The arrows heads representthe respective xenograft tumours. **B.** Representative H & E staining pictures of corresponding tumours (10X and 20X

magnification) showing necrosis under GSI treatment.**C.** Tumour growth curves after GSI and placebo treatments (both right and left flank tumours were taken into consideration).The curves clearly show the effective inhibition of tumour growth after GSI IX treatment.Bargraph representing the difference in **D.**mouse weight and **E**. tumour volume under GSI and vehicle treament for four weeks. Differences were considered as statistically significant when the P-value was<0.05 and non significant "n.s." when the P-value was>0.05. Error bars show standard deviation.**F**. Macroscopic images of control and GSI treated explanted xenograft tumours.

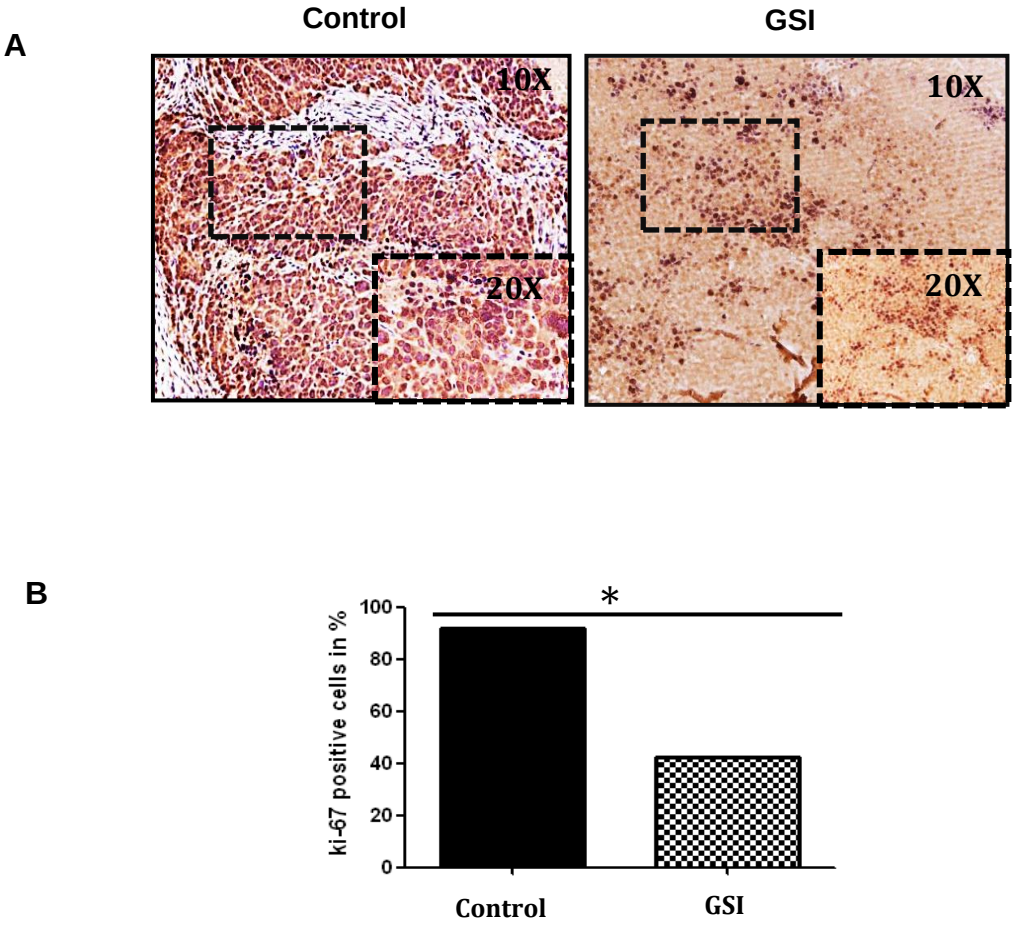


Figure 23: GSI treatment effectively inhibits the proliferative of CD44$^+$ xenograft tumours.

A. Ki67 staining images of corresponding tumours under 10X and 20X magnification, showing effective downregulation of the anti proliferative marker after GSI treatment. **B**.Graph representing the quantification of Ki67 staining of the xenograft tumours under placebo and GSI treatment(* P<0.05).

Ki67 is an anti-proliferative marker and therefore we analysedthe expression level of Ki67 by immunohistochemical staining on both GSI IX and placebo treated resected xenograft tumours. Tumours under GSI IX treatment showed considerable decrease in Ki67 expression in treated tumours as compared to the placebo group (Figure 23 Aand B). Thus, the staining clearly showed that GSI IX has a strong anti-proliferative effect on gastric tumour growth. In order to understand the target specificity of GSI IX treatment on xenograft tumours we also analysed the expression level of CD44 and Hes1 by immunohistochemistry/immunofluorescence. The stainings clearly showed a considerable decrease in expression of both the targets CD44 and HES1 under GSI IX treatment (Figure 24 A, B and C).

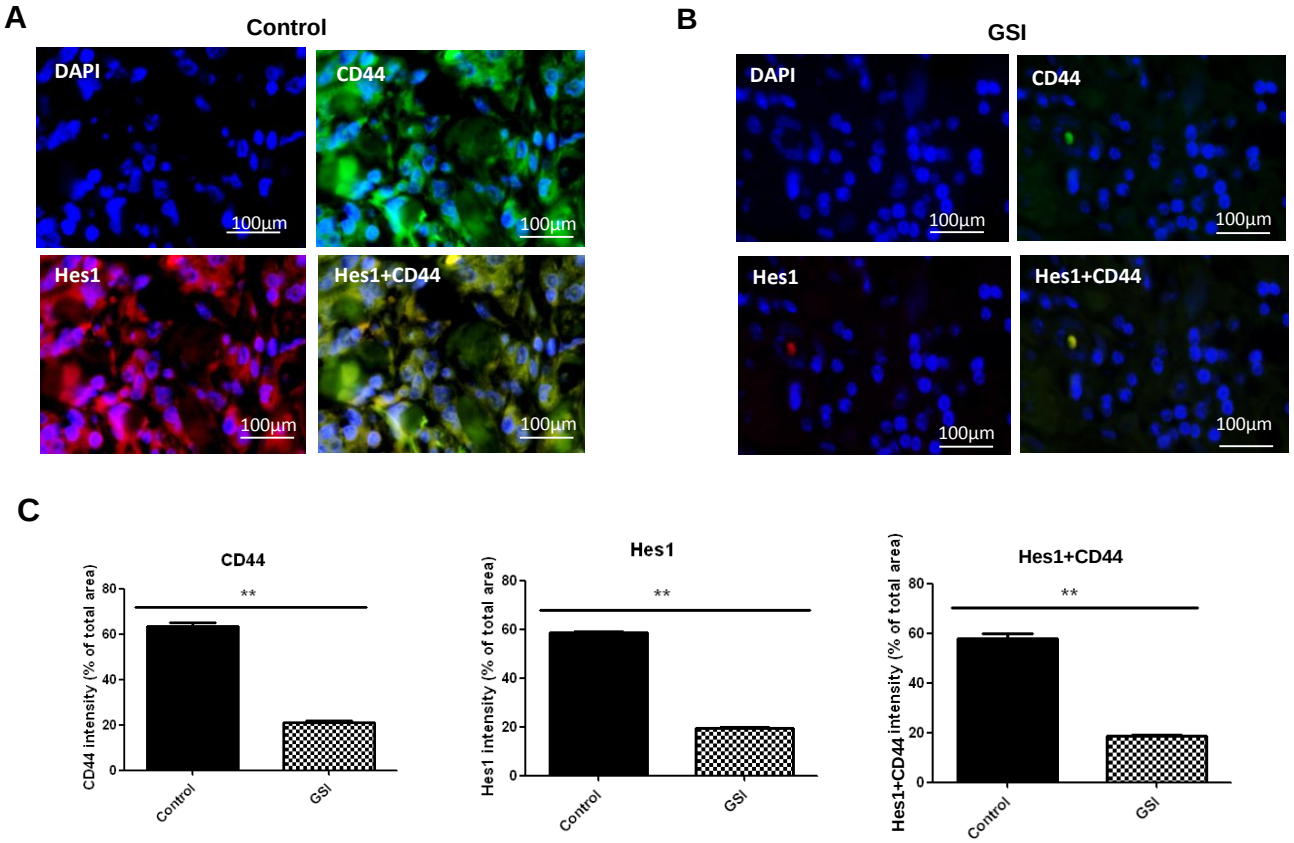


Figure 24: GSI inhibits gastric cancer xenograft tumour growth by specifically targeting the colocalised population of CD44^{+} and Hes1^{+} cells.

Representative immunofluorescence images for xenograft tumours for CD44 (green), Hes1 (red), CD44 and Hes1 merged (yellow) and 4,6-diamidino-2-phenylindole (DAPI, blue) for CD44, Hes1 stainings of **A.** Control **B**. GSI treated xenograft tumours. **C**. Graphs representing the quantification of the immnufluorescence staining intensities for CD44, Hes1, $Hes1^+$ and $CD44^+$ for placebo and GSI treated xenograft tumours. (**P<0.005)

Western blot analysis of the xenograft tumours showed an effective decrease in all the common downstream targets like CD44, Hes1, Notch1, c-Myc,wnt 5a/b, beta-catenin etc. The common downstream effector proteins involved in the crosstalk of both Notch and wnt-beta-catenin pathway in $CD44^+$ gastric cancer like p-Erk, p-Stat3, Notch, Rac1 were effectively inhibited (Figure 25A and B). Full forms of Stat3, Akt, Erk remained relatively unchanged under GSI IX treatment. For Notch pathway we checked for Hes1, Notch1, c-Myc etc by western blotting to confirm the inhibitory effect of GSI IX on Notch pathway via CD44. We also wanted to check the inhibition effect of GSI IX on wnt-beta-catenin pathway. Therefore, using western blotting we checked for some main stream and downstreams for wnt-beta-catenin pathway. Our western blot results clearly showed the effective downregulation of wnt-beta-catenin pathway downstreams like Axin2, APC and LPR6 under GSI IX treatments (Figure 25 B). Since Axin2,APC, LPR6 are involved in the formation of complex that helps in the translocation of beta-catenin to the nucleus therefore downregulation of these effector proteins shows effective inhibition of wnt-beta-catenin pathway by GSI IX inhibitor in $CD44^+$ gastric xenograft tumours. All the above mentioned inhibitory effect of GSI IX on Notch and wnt-beta-catenin crosstalk *in vivo* where in accordance with the effect observed *in vitro*. As mentioned earlier EMT is associated with tumour invasion. Using western blot we also checked the expression of EMT markers like E- cadherin and N-cadherin in xenograft tumours. GSI IX treated tumours influenced an effective upregulation of epithelial marker E-cadherin and downregulation of Mesenchymal marker N-cadherin (Figure 25B). The western blot clearly showed an effective inhibition of EMT process by GSI IX treatment in $CD44^+$CSC xenograft tumours.

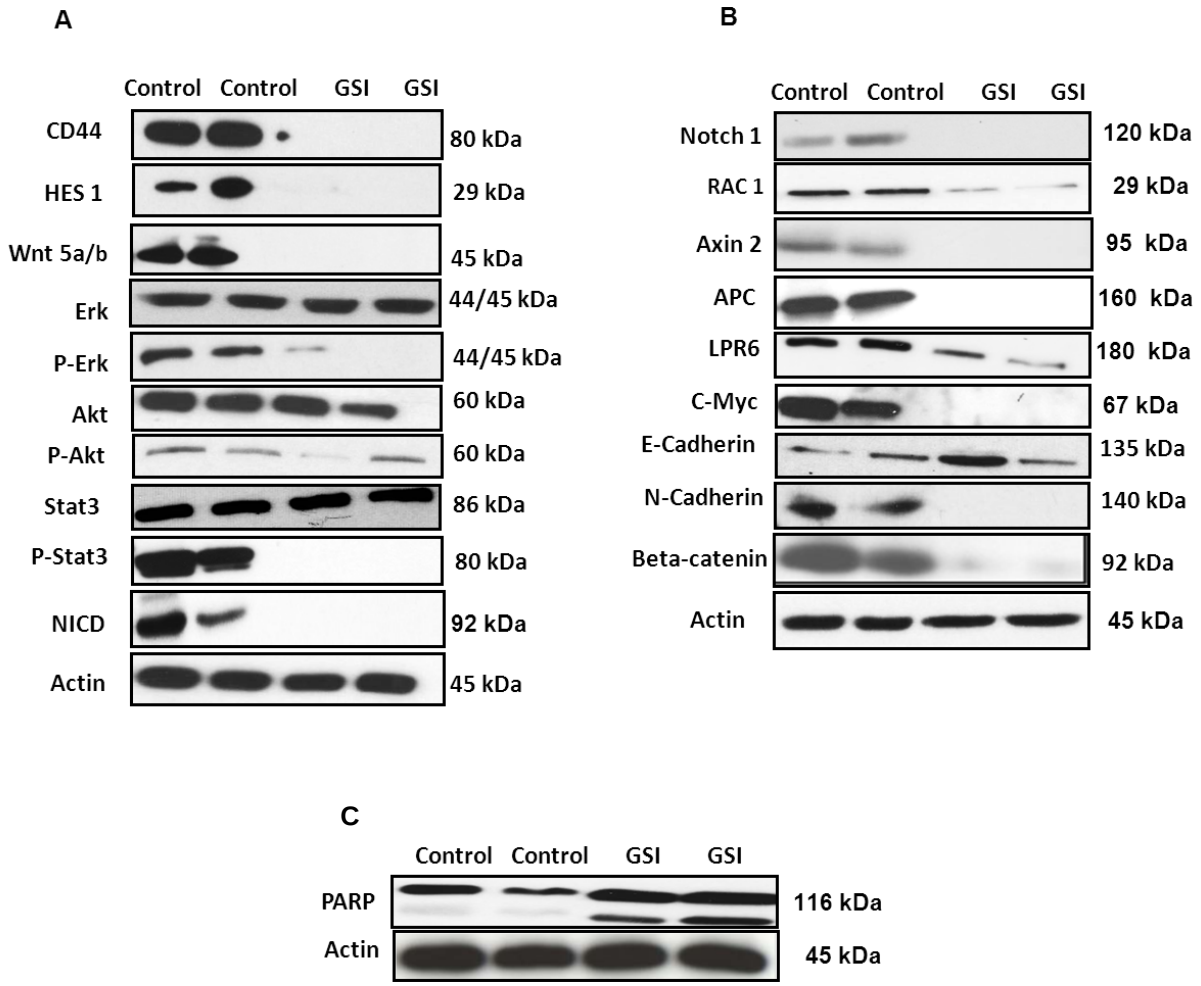


Figure 25: GSI inhibits the growth of CD44^{+} xenograft tumour growth via concomitant inhibition of Notch and wnt-beta-catenin pathway.

A.The expression of CD44, Hes1, wnt 5a/b, Erk, p-Erk, Akt, p-Akt, Stat3, p-Stat3 and NICD of GSI and placebo treated xenograft tumours by western blotting. **B**. Notch1, Rac1, Axin2, APC, LPR6, c-Myc, E-cadherin, N-cadherin and beta catenin expression levels were also analysed by western blots for GSI and placeo treated tumours. Beta-actin was used as a loading control. **C**. Cleavage of PARP was also analysed using western blot for GSI and placebo treated xenograft tumours.Actin was used as a loading control.

To reconfirm the induction of apoptosis by GSI IX treated xenograft tumours as observed *in vitro*, we performed western blot analysis of PARP. Immunoblot showed cleavage of PARP in GSI treated tumours as compared to the control or placebo treated xenograft tumours (Figure 25 C). Therefore, GSI IX also induces considerable cell death in xenograft tumours which is in accordance with our *in*

*vitro*findingsthat also highlights induction of cell death involved in GSI IX mediated treatment. Thus, our results clearly supports that GSI IX treatment effectively inhibits the concomitant activation of both the pathways Notch and wnt-beta-catenin by targeting CD44 in $CD44^+$ CSC xenograft tumours. Moreover,our*in vivo* data also supports induction of apoptosis by GSI IX treatment similar to *in vitro* data.

4.9$CD44^+$ and $Hes1^+$ double positive cells could be a new target for gastric cancer.

CD44 is also used as a cell surface marker for early T cell development in the thymus and also for tracking memory T cells.Notably, Hes1 is also found to be involved in the development of T cells (17). To establish CD44 as a CSC marker for gastric cancer we selected $CD44^+$ alongwith $HES1^+$ as a double positive marker to specifically target gastric CSC. Usage of double positive marker ($CD44^+$ and $Hes1^+$) also reduces the chance of targeting CD44 present in normal human blood. Therefore, in order to understand the specificity of GSI IX treatment *in vivo*on $CD44^+$ CSC xenograft tumours we performed FACS analysis using mononuclear cells isolated from the blood collected from both the groups (Control and GSI IX treated xenografts). The FACS analysis clearly showed an effective downregulation under GSI IX treatment of the double positive ($CD44^+$ and $Hes1^+$) cells in xenograft blood (35 to 40%) compared to the control/placebo group blood (75 to 80%) (Figure 26 B and C). Moreover, we also compared the percentage / incidence rate of ($CD44^+$ and $Hes1^+$) in the blood of normal mice to that of $CD44^+$ CSC xenografts (Figure 26 A). This was done in order to reduce the chances of off target effects on the normal population of $CD44^+$ and $Hes1^+$ cells expressed in normal blood due to the presence of T lymphocytes. FACS analysis of monocytes isolated from the blood of a normal mice (without tumour disease)revealed that only 20% of cells were found to be $CD44^+$ and $Hes1^+$ double positive. This suggests that the GSI IX treatment shows a probable specific approach only towards $CD44^+$ and $Hes1^+$ double positive cells as the average count of double positive cells in normal blood is same as that observed in the blood of the GSI IX treated xenografts (Figure 15D). Notably blood from human patients as well as control xenografts showed a relatively higher expression of $CD44^+$and $Hes1^+$ double positive cells which highlights that $CD44^+$ and $Hes1^+$double positive cells can be an attractive target for GSI IX treatment.

Moreover, the decrease in the expression of $CD44^+$ and $Hes1^+$ double positive cells shows specificity for GSI IX treatment. Importantly, the average count of $CD44^+$ and $Hes1^+$double positive cells remains constant as observed in the blood of normal mice to that of GSI IX treated xenografts.

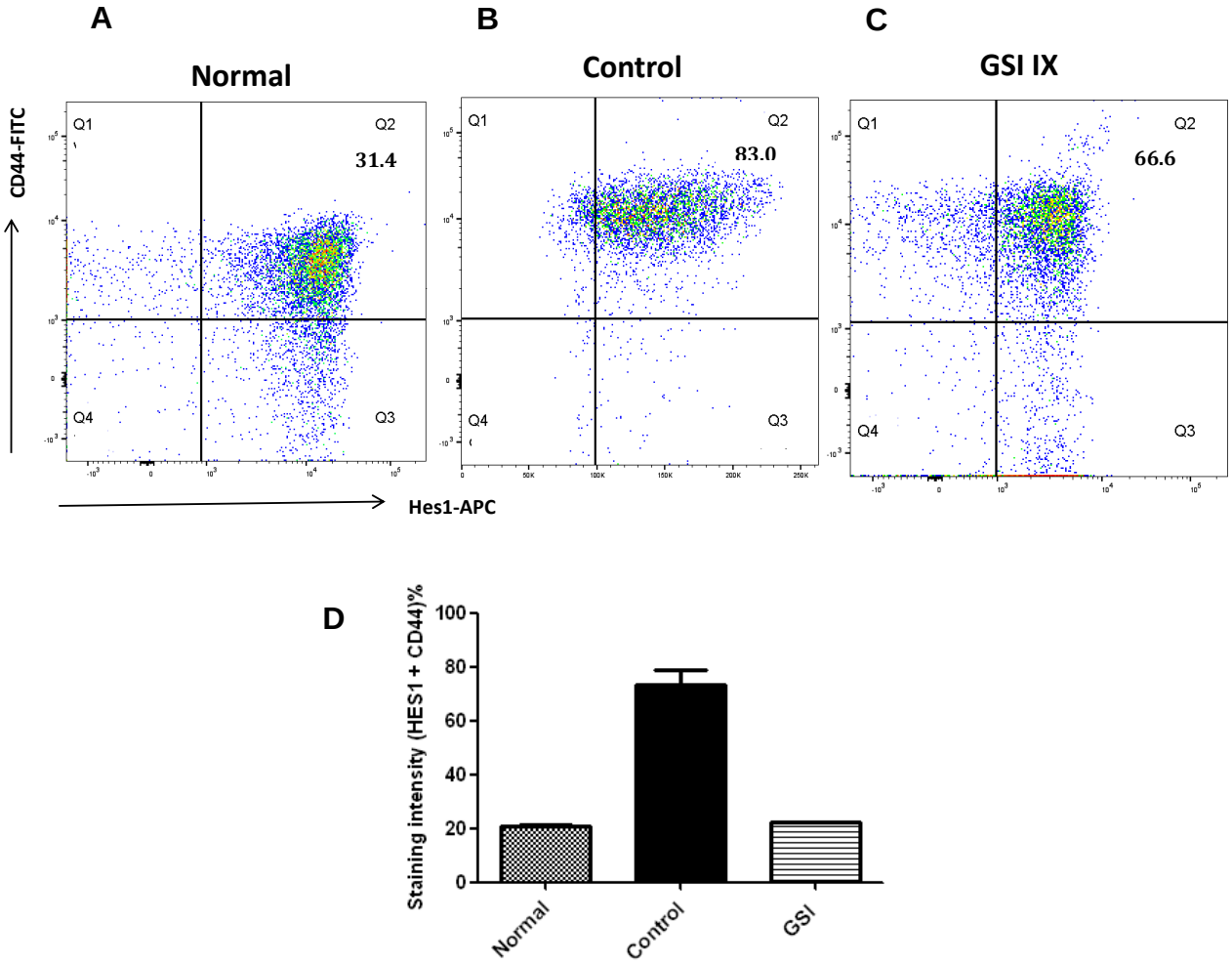


Figure 26: GSI inhibits CD44 and Hes1 double positive in $CD44^+$ xenografts.
FACS analysis of double positive cells ($CD44^+$ and $Hes1^+$) in monocytes isolated from **A**.Normal mice **B**. Placebo treated and **C**.GSI treated $CD44^+$ xenografts (blood from C57BL/6 mice were used for normal mice). **D**.Graph representing the quantification of $CD44^+$ and Hes1 double positive cells analysed by FACS in blood collected from three different groups (Normal, control and GSI) mice.

We also wanted to check the expression of $CD44^+$ and $Hes1^+$ double positive cells in the blood of gastric cancer patients in comparison to normal individuals. FACS

analysis of mononuclear cells isolated from the blood of both normal and gastric cancer patients showed a significant (P<0.005) increase of double positive cells in the blood from gastric cancer patients (65 to 70%) in comparison to that of normal individuals (20 to 22%) (Figure 27 A, B and C). Therefore, the FACS analysis of human blood clearly potentiates the effectiveness or the possiblity of using $CD44^+$ and $Hes1^+$ double positive cells as an attractive target for gastric cancer treatment.

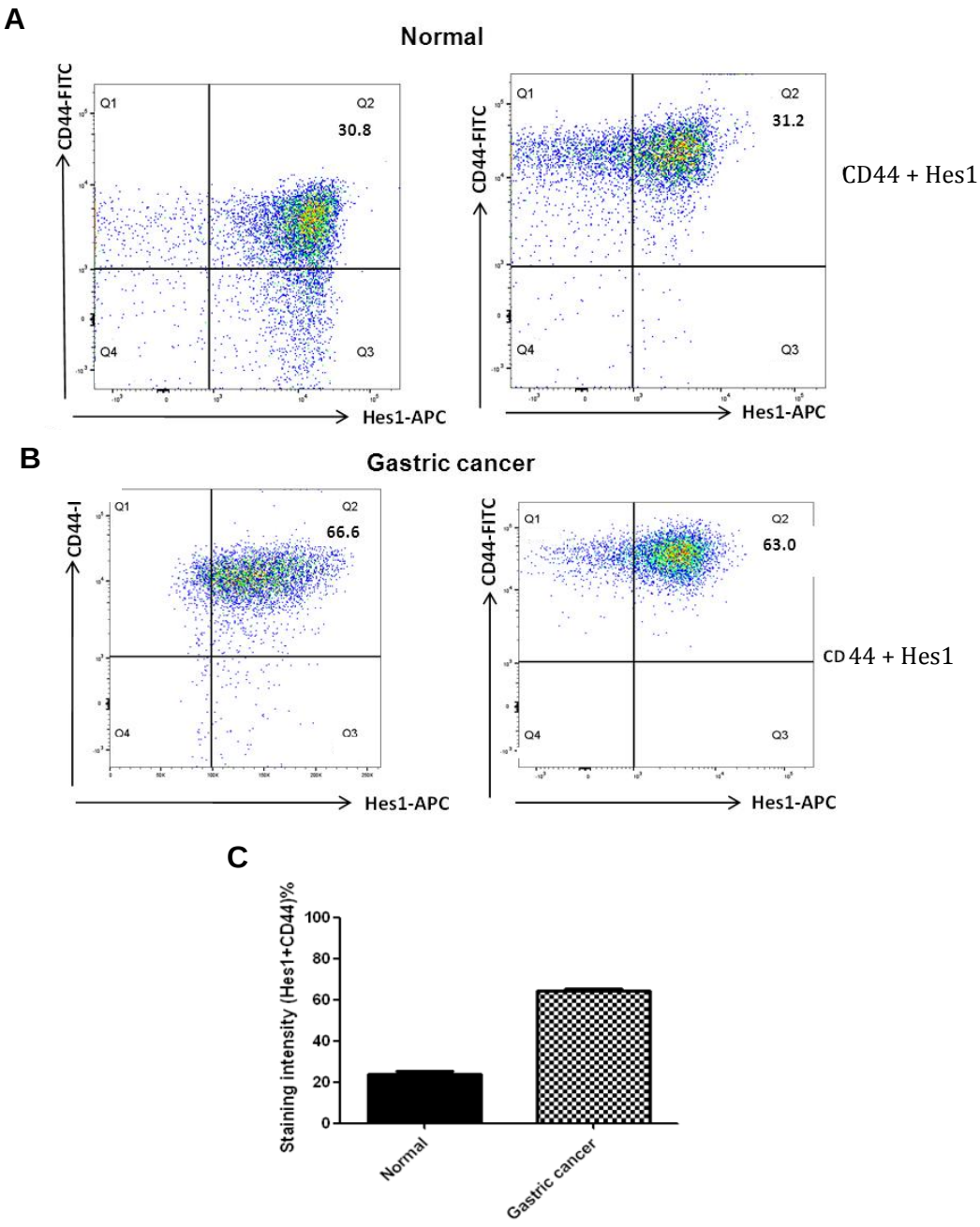


Figure 27: Expression levels of $CD44^+$ and $Hes1^+$ double positive are higher in gastric cancer patients compared to normal individuals.

FACS analysis showing the percentage of double positive cells ($CD44^+$ and $Hes1^+$) in the mononuclear cells of blood isloated from **A**. Normal healthy individuals and **B.** Gastric cancer patients. **C**. Graph representing the quantification of the relative percentage of expression of CD44 and Hes 1 double positive cells in the mononuclear cells of blood collected from patients (gastric cancer patients vs normal individuals) (**P<0.005).

Taken together, FACS analysis data clearly shows $CD44^+$ and $Hes1^+$ double positivity as a probable futuristic marker for gastric cancer treatment. The decrease in double positives ($CD44^+$ and Hes1) under GSI IX treatment highlights these cells as an attractive target for GSI IX mediated treatment of $CD44^+$ CSC in gastric cancer.

Therefore, our *invitro* and *invivo* results suggest a strong anti cancer effect of GSI IX on gastric cancer. A strong correlation between *in vivo* and patient data reflects $CD44^+$ and $Hes1^+$ double positive cells as a probable futuristic marker for gastric cancer treatment. GSI IX effectively targets $CD44^+$ CSCs and inhibits gastric cancer growth via concomitant blockage of Notch and wnt-beta-catenin pathways. Thus, GSI IX proves to be an effective, target specific therapeutic option for gastric cancer.

Discussion and Outlook

5. Discussion and outlook

5.1 Targeting CSCs for gastric cancer treatment

CSCs are one of the primary candidates involved in tumour relapse and resistance to chemo or radiotherapy (54). Targeting CSCs using specific cell surface marker to inhibit CSC mediated resistance of tumour cells has been a debatable approach in the field of cancer treatment. Therefore, selective targeting of CSC population could be of immense interest in cancer cure. Moreover, identification of CSC using specific cell surface markers has been an effective tool towards targeting these candidates of tumour resistance (55).

CD44 is widely recognised as a pleotropic molecule expressed on the surface of stem cells and is also known to be involved in tumour metastasis. Thus, targeting CSC using CD44 as a cell surface marker could be an effective way towards CD44 associated tumour growth (54, 55) .

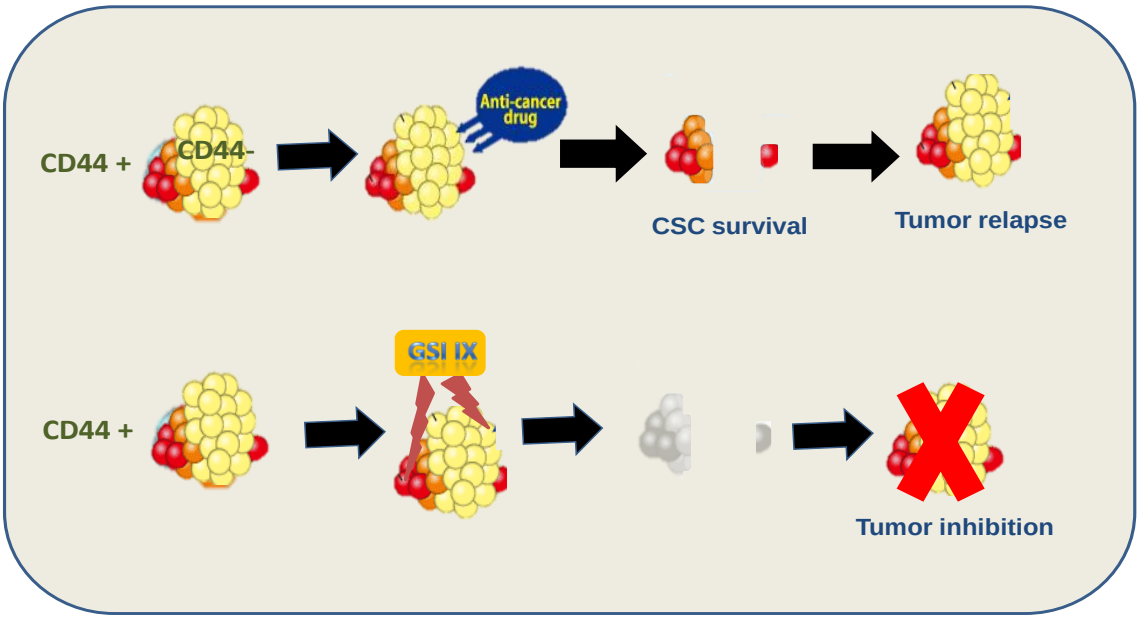


Figure 28:Targeting CD44 gastric cancer initiating cells via GSI IX (Gamma secretase inhibitor IX) to inhibit gastric cancer.

The diagram showing the tumour relapse with general anti cancer drugs in comparison to CD44 target specific inhibitors like GSI IX thus leading to complete inhibition of tumours (adapted from 81).

Several signaling pathways like Notch, Hedgehog,wnt-betacatenin etc has been identified as activators of CD44 receptor (60, 61, 74, 75). Important to our present study CD44 levels has been identified to be upregulated in a wide variety of cancers like pancreatic, breast, colon and also in gastric cancer (84, 85). CD44 overexpression was found to be associated with poor prognosis and lower survival in $CD44^+$ gastric cancer patients in comparison to the $CD44^-$ population (86). In addition to gastric cancer, studies by Hong et al.,2009 (87) showed that in pancreatic cancer patients CD44 expression was associated with histological grade and poor prognosis (86). The role of CD44 in chemotherapeutic resistance was always debatable due to conflicting results obtained in different studies (87, 89, 94). Studies showed that metastatic colorectal cancer patients treated with oxaliplatin or 5FU (standard chemotherapies used for cancer treatment) showed no strong correlation between CD44 expression and chemotherapeutic resistance or overall patient outcome (35). In contrary to the previous study, *in vitro* colon cancer cells showed a significant decrease in CD44 expression under treatment with oxaliplatin or 5FU and also incombination with curcumin (88). Moreover, Jaggupilli A et al.,2012 (90) showed expression of CD44 in colonic tumours thus, reflecting CD44 as one of the main candidates associated with tumour growth and metastasis. Interestingly, increased proliferation of $CD44^+$ pancreatic cancer cells upon gemcitabine treatment also showed a strong correlation between CD44 expression and chemotherapeutic resistance(89, 87). Overall, these studies reflect an important role of CD44 in cancer progression, and it also potrays that the dual contradictory role of CD44 is highly dependent on the cancer types.

Gastric cancer is known to be the second most common cause of cancer related death worldwide. New modes of gastric cancer treatments are of utmost need. Our study mainly potentiates targeting of CD44using gamma secretase inhibitor IX (GSI IX) as an effective approach in gastric cancer treatment. Though CD44 is known to play a contradicting role in colon cancer yet regarding gastric cancer development CD44 has also shown to be an effective marker associatedwith CSC mediated cancer progression (91, 51). GSI IX has already been assesed as a potential inhibitor in several cancers including pancreatic cancer etc. Studies by Palagani et al.,2012 showed that$CD44^+$/ $EpCAM^+$ pancreatic cancer cells are inhibited by GSI

and the decrease of $CD44^+$ cells correlates with response to chemotherapy in a panel of gastrointestinal cancer (76, 93).

In this current study we validated the role of CD44 in gastric cancer and also showed GSI IX as an effective therapeutic treatment option to target $CD44^+$ CSC. CD44 mediated CSC maintenance is regulated by some of the major cancer associated pathways. The role of Notch in gastric cancer has been explored for many years. Amongst various pathways involved in CSC maintenance Notch and wnt-beta-catenin pathways are found to be the two most important pathways associated with CSC mediated cancer progression (94, 95). However, the involvement of CD44 and Notch signaling as a new target in gastric cancer progression and specific targeting of $CD44^+$ CSCs by GSI still remained to be explored as an alternative therapeutic approach in gastric cancer.

5.2 CD44, Hes1 as a prognostic marker for gastric cancer patients

We first studied the expression of CD44 in a panel of 269 gastric cancer patients and correlated the expression of CD44 to overall patient survival. Patients with CD44 and Hes1 double positivity showed an overall impaired survival in comparison to CD44 and Hes1 negative population. Survival analysis identified the potential role of CD44 and Hes1 double positivity in gastric cancer prognosis.Our data also demonstrated a significant expression of CD44 and Hes1 in tissue and blood samples of gastric cancer patients. Considering the role of wnt pathway in CD44 mediated CSC maintenance, we also analysed the expression of wnt 5a/b in gastric cancer patient samples. Interestingly, effective co-activation/co-expression of wnt 5a/b was also observed in the same samples showing upregulation of both CD44 and Hes1. Therefore, activation of both Notch (Hes1) and wnt (wnt 5a\b) pathway in $CD44^+$ gastric cancer patient samples was also observed. Sequential increase in expression of the three targets (CD44, Hes1, wnt 5a/b) was specifically observed being highest in gastric tumours in relation to inflammation or normal patient samples. Additional analysis of patient blood in comparison to normalindividuals also reflected an upregulation of CD44 and HES1 double positivity from being 22% in normal to 66% in gastric cancer patients. Thus,analysis of patient samples mainlyfoccussed on the prognostic role of Hes1 and CD44 double positivity as an effective marker.

Additionally, existence of concomitantly active Notch and wnt pathway in $CD44^+$ gastric cancer patient tumours was also verified.

5.3 Inhibition of Notch pathway in $CD44^+$ CSC in gastric cancer

Inhibition of Notch has been regarded as an effective therapeutic strategy in various cancer studies. Previous studies have shown effective inhibition of Notch pathway in pancreatic cancer cells by GSI IX (90). Amongst the various novel strategies available for gastric cancer treatment, inhibition of Notch pathway is also considered as an important target. Studies have shown that alteration of Notch pathway led to the development of various human malignancies including gastric cancer (96, 97, 98). Therefore, we tested the inhibitory effect of GSI IX in $CD44^+$ MKN45 cells to show that Notch signaling might contribute to the maintenance of $CD44^+$ gastric CSC. MKN45 cells reflected diffuse type of gastric cancer as it is established from poorly differentiated, diffuse type adenocarcinoma of the stomach (65). GSI IX treatment showed effective inhibition of all important cell regulatory processes associated with cancer growth like proliferation, migration, invasion, adhesion (CD44 also acts as an important cell adhesion molecule involved in tumour progression) and tumour sphere formation (important characteristic feature of CSC) in $CD44^+$ MKN45 cells in a time and dose dependent manner. Further coactivation of CD44 and Hes1 in the same population of $CD44^+$ MKN45 cells was observed. Importantly, effective inhibition of Hes1 via targeting of CD44 under GSI IX treatment in $CD44^+$ gastric CSCs was achieved. GSI IX treatment also showed effective induction of apoptosis as a key role mechanism to induce cell death in $CD44^+$ gastric CSC. These results clearly highlighted inhibition of Notch signaling by GSI IX as a potential treatment approach for $CD44^+$ gastric cancer.

5.4 GSI might influence Epithelial-to-mesenchymal transition (EMT) via RAC1 in $CD44^+$ CSC

EMT is an important collective event which results in conversion of epithelial adherent cells to independent fibroblastic cells possesing migratory or invasive properties (54). Previous studies have shown contribution of Notch signaling to acquisition of EMT processes (84). Interestingly, GSI IX treatmentshowed a low or partial inhibitory effect on EMT markers like E-cadherin, N-cadherin etc.

Incontrary,GSI IX effectively inhibited EMT associated processes like migration and invasion in $CD44^+$ MKN45 cells. This contradiction can be attributed to the role of RAC1 played in modulation of epithelial cell plasticity in cancers through matrix metalloproteinases (MMPs). Since GSI treatment showed effective inhibition of RAC1 thus it can be assumed that probablyGSI mediates/ regulates EMT via RAC1 through MMPs(99). Threfore, GSI mediated inhibition of epithelial plasticity in gastric cancer through MMPs could be of futuristic importance. Moreover, this strategy could also open up new ways of EMT inhibition through MMPs in gastric CSCs, but further investigations are required for confirmation of this proposed alternative.

5.5 Dual inhibition as an effective strategy for gastric CSC

Abberant activationof wnt beta-catenin pathway hasbeen found to be associated with certain areas of gastric cancer progression and is also known to play important role in regulating tumour growth, metastasis etc. Importantly, a strong correlation was observed between wnt1, CD44 expression and grade of gastric cancer patients (100, 101). Moreover, important to our study Western blot analysis of the $CD44^+$ MKN45 showed a similar activation of both Notch and wnt along with CD44 in accordance with the findings of the patient's samples (102, 103). Therefore, we further analysed and showed concomitant activation of both Notch and wnt-beta-catenin pathway in CD44 positive MKN45 cells. Moreover, we also further highlighted on GSI IX mediated dual inhibition of both Notch and wnt betacatenin pathways in these $CD44^+$ gastric cancer cells. GSI IX treatment not only showed effective inhibition of Notch pathway and its downstreams like Hes1, Notch 1 etc but also effectively inhibited the wnt-beta-catenin pathway via downregulation of common wnt downstreams like LPR6, Axin2, wnt 5a/b etc. Therefore, our findings firstly focussed on the activation of Notch and wnt-beta-catenin pathway in $CD44^+$ gastric CSCs. Secondly, we also proposed in this study the existence of a probable crosstalk between Notch and wnt-beta-catenin signaling cascades mediated by CD44 (using some common downstream effector proteints like c-Myc, Akt, Erk etc associated with important cell regulatory processes resulting in cancer progression) in $CD44^+$ gastric CSCs. Finally, we also identified GSI IX as a potential drug which can concomitantly inhibit both the pathways (Notch and wnt-beta-catenin) associated with gastric CSC maintenance via effective targeting of CD44. Therefore, reducing all chances of

tumour relapse or resistance to standard chemotherapeutic regimes associated with CSCs.

5.6 Notch1 might influence important crosstalks in CD44$^+$ CSC

Several studies have shown an effective or critical role of Notch receptors in gastric cancer development. Yeh et al.,2004 (104)showed that Notch 1 activaton leads to the development of COX2 dependent gastric cancer progression(34). Moreover, study by Yeh et al.,2009(104)demonstrated the role of Notch 1 in gastric CSC regulation (103). In contrast to the mentioned studies Notch4 has also been attributed to play an important role in gastric carcinogenesis (105). Qian et al.,2015 (106) reported an important role of Notch 4 involved in the activation of wnt-beta-catenin pathway in gastric cancer (106). Furthermore, Kang et al., 2012 (107) described the expression of Notch3 and Jagged2 in association with GC development (107). Overall, these studies highlights the contradictory importance of Notch receptors (Notch 1, 2 3 and 4) in gastric cancer progression. However, our results demonstrated the role of Notch 1 in CD44 mediated gastric CSC maintenance. The western blot analysis showed Notch 1 to be an effective and important mediator of Notch and wnt-beta-catenin crosstalk in CD44$^+$ gastric CSC. siRNA mediated silencing of CD44 and Notch 1 further strengthened the important regulatory role played by Notch 1 in CD44 mediated maintenance of gastric CSC via activation of Notch and wnt-beta-catenin pathways. Notch1 as well as Notch2 are already known to be critical for the metaplastic transition of gastric epithelial cells (108). Hence, our study represented a significant extension of theprevious studiesin gastric cancershowing that Notch1 is the main target and critical for the interaction between Notch signaling and GC CD44$^+$ cells. Our findings clearly showed Notch 1 dependent interaction in CD44$^+$ gastric CSCs compared to Notch 2 or 3 specifically under GSI treatment. In addition, silencing of CD44 and Notch1 resulted in a significant downregulation of multiple target genes that could play a regulatory role in exhibiting the Notch 1 mediated dual crosstalk in gastric CSCS.

Amongst colon, breast and lung tumours overexpression or mutation of c-Myc has also been observed in gastric cancer. However the role of c-Myc in gastric carcinogenesis needs to be explored more in detail (109). c-Myc known to be an important oncogene and relative to our study, a direct downstream of Notch1

(109,110, 111) might also be important in mediating the crosstalk by acting as an effective downstream effector protein connecting CD44 and Notch, wnt-beta-catenin pathways in gastric CSCs. Sawant S et al.,2016 showed an effective independent as well as combinational role with CD44 in oral cancer (112). Moreover, a regulatory reciprocal role has also been observed between CD44 and Epidermal Growth Factor Receptor (EGFR) via c-Myc in gastric cancer (110). Contrastingly GJ Yoshida etal.,2014showed an inverse realtionship between CD44 and c-Myc in gastric cancer cells (111). Our western blot analysis showed c-Myc as an effective connective effector for the crosstalk followed by effective inhibition under GSI IX treatment. Moreover, silencing of CD44 also showed a direct regulatory impact on c-Myc expression in gastric CSC. Therefore, all together c-Myc might act as a connective effector between CD44, Notch1 and wnt-beta-catenin pathway in gastric cancer.

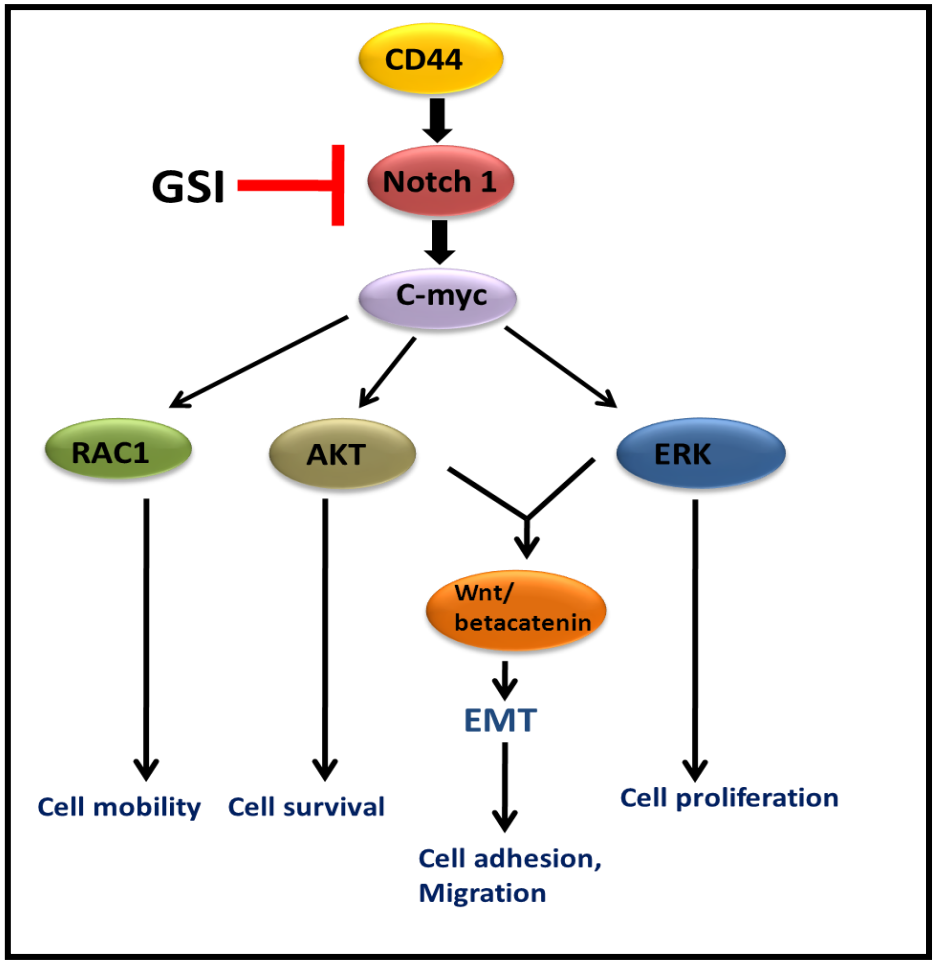


Figure 29: Schematic representation of GSI IX mediated dual inhibition of Notch and wnt-beta-catenin pathways in CD44$^+$ gastric cancer.

Diagram representing the concomitant inhibition of Notch and wnt-beta-catenin pathway in CD44+CSCs by GSI IX treatment. Notch1 impairment by GSI IX leads to inhibition of c-Myc (a direct downstream of Notch1). This in turn causes inactivation of both the pathways (Notch and wnt-beta-catenin) via inhibition of common downstreams. We also hypothesised that this crosstalk is on a whole mediated by CD44. Thus targeting of both Notch and wnt-beta-catenin pathway active in CD44+CSCs, is effective for GSI IX treatment.

Important cell regulatory downstream target genes like pAKT which plays a key role in multiple cellular processes such as glucose metabolism, apoptosis, cell proliferation, transcription and cell migration was altered (112). Additionally, Mitogen activated protein / extracellular signal-regulated kinase (MAP/ERK) pathway, and STAT3 (signal transducer and activator of transcription 3) associated with important role in control of cellular processes like proliferation, survival, differentiation and motility etc were effectively impaired (114, 115). Furthermore, RAC1 (Ras-related C3 botulinum toxin substrate), a protein important for cell motility and cell growth is downregulated under GSI therapy (115). GSI treatment was able to impair important target genes, which all have leading functions or roles for cellular processes. As a new aspect, our data additionally showed GSI IX mediated effective inhibition of a cross talk between Notch and wnt-beta-catenin pathway via Notch1 in gastric $CD44^+$ CSCs and indicated these two pathways as attractive targets for GSI mediated therapy. CD44 and specifically Notch1 silencing showed us an overall effective, similar downregulation of thecommon downstreams as achieved by GSI IX. Thus, these findings focusses on Notch1 in mediating the crosstalk and also highlights the importance of GSI IX treatment in gastric cancer . It also further strengthens that GSI targets $CD44^+$ CSCs via inhibition of Notch1 which in turn helps in the concomitant inhibition of Notch / wnt-beta-catenin pathway. Our study revealed a new effective dual inhibitory approach for CSC mediated gastric cancer treatment.

5.7 GSI IX impairs gastric CSC *invivo* via targeting $CD44^+$ and $Hes1^+$ double positive prognostic cells

In accordance with our *in vitro* studies*in vivo*growth of $CD44^+$ xenograft tumours was also inhibited under GSI IX treatment. Moreover, western blot analysis showed a consistant effective inhibition of the Notch wnt beta-catenincrosstalk after GSI IX treatment in these tumours. Importantly, FACS analysis using xenograft blood not

only revealed a strong connection between gastric cancer patients and xenografts reflecting the presence of double positive cells as a probable futuristic marker for gastric cancer prognosis but, additionally GSI IX treated xenografts showed an effective downregulation of these double positive ($CD44^+$ and $Hes1^+$) cells in comparison to the normal. Therefore, these results indicates GSI IX treatment as an effective therapeutic option to enhance the prognostic value and inhibit these double positive cells associated with gastric cancer development. Notably, the average count of double positive cells were maintained to the basal level under GSI IX treatment similar to that observed in normal individuals due to presence of CD44 (CD44 is expressed in leucocytes and normal red blood cells)(117) and Hes1 (Notch and Hes1 expression is important for haematopoetic lineage in normal blood) (118) positive cells in normal blood. This indicates that GSI IX effectively inhibits the double positive gastric CSCs specifically without inhibiting the expression of these targets in normal individuals.

5.8 GSI IX is a target specific drug against $CD44^+$ CSCs

To mitigate the off target toxic side effects of GSI IX treament and also to enforce the target specificity of GSI treatment reducing the chances of tumour relapse, we showed a comparitive analysis using proliferation assay of GSI IX treatment to that of 5Flurouracil (5FU). 5FU is a strong, standard chemotherapeutic regime used for gastric cancer treatment. 5FU treatment showed partial inhibition of both $CD44^+$ and $CD44^-$ MKN45 cells proliferation whereas GSI IX treatment effectively inhibited and targeted $CD44^+$ CSC population of MKN45 cells.Therefore, GSI IX treatment proved to be more effective and target /site specific in action as it particularly inhibits $CD44^+$ CSCs in comparison to 5FU which fails to specifically inhibit $CD44^+$ gastric CSCs and also exerts off target effects on $CD44^-$ population of gastric CSCs. GSI IX treatment therefore effectively removes all the boundaries associated with targeting CSC for cancer treatment and also hinders or blocks the chances of tumour relapse and resistancy of chemotherapies to gastric CSC. This is achieved by effective inhibition of both CSC associated pathway or feedback loop resulting in complete inhibition of gastric CSC mediated cancer progression.

5.9 Beneficial facts of our study

Recently a study by Li et al.,2015 (119) also tested the inhibitory effect of GSI on $CD44^+$ MKN45 cells both *in vitro* and *in vivo* similar to our present approach but their study differed in few aspects. The authorsdid not use more than one drug concentration of GSI and also lacked analysis of human data (119). Moreover, they showed considerable change of EMT markers like E-cadherin, N-cadherin, Vimentin etc in contrary to our study. Finally, Li and his colleagues in their study focused only on Notch1 signaling pathways unlike us as we check on both Notch and wnt-beta-catenin pathway. However, using three different dosages of this drug our data showed an effective approach by achieving partial to complete inhibition in GSI IX therapy. We also highlighted the prognostic value of $CD44^+$ and $Hes1^+$ double positivity, importantly also revealed a strategy of GSI IX mediated inhibiton of Notch and wnt-beta-catenin pathway crosstalk involved in CD44 CSC exhibited gastric cancer progression. These additional findings prove our study to be of immense therapeutic interest with potential futuristic aspects for gastric cancer treatment specifically in the field of CSC in comparison to the previously published reports.

5.10 Highlights and futuristic aspects of our study

To summarise, for the first time our study shows the colocalisation of CD44 and Hes1 in $CD44^+$ gastric CSC. We also highlight not only the colocalisation and activation of Notch signaling pathway (Hes1 is a direct downstream of Notch) in $CD44^+$ CSCs, but also revealed the concomitant activation of wnt-beta-catenin pathway along with Notch in $CD44^+$ gastric CSCs. Thus, we showed the importance of dual inhibition using single agent therapy GSI IX in these $CD44^+$ gastric CSCs. Dual inhibition of both Notch and wnt-beta catenin pathway in $CD44^+$ gastric CSC was achieved using GSI treatment which not only effectively inhibited both the pathways associated with CSC maintenance but also strongly blocked the chances of tumour relapse, which might occur due to the existence of feedback loop in between these two pathways in gastric cancer. We also underlined the importance of $CD44^+$ and $Hes1^+$ as a prognostic marker in gastric cancer. CA19-9 is a widely accepted tumour marker for gastric cancer with prognostic importance(120). Therefore, for the first time we show the potential of $CD44^+$and $Hes1^+$ double

positive cells as a prognostic marker for gastric cancer. GSI IX mediated therapy can be specifically implemented to the group ofpatients showing $CD44^+$ and $Hes1^+$ double positivity as these cells also reflect the activation of the Notch pathway as a major contributor for CD44 maintenance. So this proves anti Notch treatment like GSI IX as an effective option for this selective cohort of patients. Moreover, we also addressed GSI IX mediated effective inhibition of these double positive cells with prognostic value in $CD44^+$ gastric CSC. The results from our study have significant implications for the treatment of gastric cancer. We also propose a probable mechanistic approach of GSI IX mediated treatment in gastric CSC but additional experiments are needed to determine our findings in a prospective study of gastric cancer patients. Clinical trials using GSI IX as a probable therapeutic approach in gastric cancer patients could be of immense interest as it can open up new avenues for treatment of human gastric cancer .

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Contributions

Contributions

Prof. Dr. Ruben Plentz contributed for study concept and design of the project, interpretation of clininical and experimental data, drafting of the manuscript.

Chen Xi and Bui Khac Cuong contributed with the injection of CD44+ MKN45 cells in the nude mice (NMRI-nu\nu) mice. Prezymslawa Bozko was involved in the interpretation of the data.

Julian Göetze contributed in collecting Blood samples from normal individuals.
Facs core facility, unikliniken Berg helped in sorting the MKN45 cells for CD44 positive population.

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