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Characterization of the involvement of cancer stem cells in gastric cancer resistance to chemotherapy

Dissertação apresentada à Universidade de Coimbra para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Celular e Molecular, realizada sob a orientação científica do Professor Doutor Filipe Santos Silva (IPATIMUP) e supervisão do Professora Doutora Emília Duarte (Faculdade de Ciências e Tecnologia da Universidade de Coimbra)

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Table of contents

Abbreviations

5-FU	5-Fluorouracil
ABC	ATP-binding cassette
ADM	Doxorubicin
AML	Acute Myeloid Leukemia
BCA	Bicinchoninic acid
Bcl-XL	B-cell lymphoma-extra large
Bcl-2	B-Cell CLL/Lymphoma 2
bFGF	Basic Fibroblast Growth Factor
BSA	Bovine Serum Albumin
Cape	Capecitabine
CD44s	CD44 standard
CD	Cluster of Differentiation
CDH1	Cadherin 1
CDDP	Cisplatin
CF	Cisplatin and 5- Flourouracil
CSCs	Cancer Stem Cells
DHFU	Dihydrofluorouracil
DISC	Death inducing Signalling Complex
DNA	Deoxyribonucleic acid
Doc	Docetaxel
DPD	Dihydropyrimidine Dehydrogenase
DTT	Dithiothreitol
EAP	Etoposide, Adriamycin and Cisplatin

ECF	Epirubicin, Cisplatin and 5- Flourouracil
ECX	Epirubicin, Cisplatin and Capecitabine
EDTA	Ethylenediamine tetraacetic acid
EEP	Epidoxorubicin, Etoposide and Cisplatin
EFP	Etoposide, 5- Flourouracil and Cisplatin
EGF	Epidermal Growth Factor
ELF	Etoposide, Leucovorin and 5- Flourouracil
EOF	Epirubicin, Oxaliplatin and 5- Flourouracil
EOX	Epirubicin, Oxaliplatin and Capecitabine
EPI	Epirubicin
FADD	FAS-Associated Death Domain protein
FAM	5- Flourouracil, Adriamycin and Mitomycin-C
FAMTX	5- Flourouracil, Adriamycin and Methotrexate
FAMe	5- Flourouracil, Adriamycin and Methyl lomustine
FAP	5- Flourouracil, Doxorubicin and Cisplatin
FAS-L	Fas Ligand
FBS	Fetal Bovine Serum
FcR	FcR Blocking Reagent
FdUTP	Fluorodeoxyuridine triphosphate
FdUMP	Fluorodeoxyuridine monophosphate
FITC	Fluorescein isothiocyanate
FLEP	5- Flourouracil, Leucovorin, Epirubicin and Cisplatin
FUMP	Fluorouridine Monophosphate

GI50	50% Growth Inhibition concentration
GIST	Gastrointestinal Stromal Tumours
HA	Hyaluronan
H. pylori	*Helicobacter pylori*
HRP	Horseradish Peroxidase
IARC	International Agency for Research on Cancer
LSC	Leukemic Stem Cell
LV	Leucovorin
MACS	Magnetic Cell Sorting
MAGIC	Medical research council Adjuvant Gastric Infusional Chemotherapy
MALT	Mucosa-Associated Lymphoid Tissue
MDR1	Multidrug Resistance Transporter 1
MMC	Mitomycin C
MMPs	Matrix Metalloproteinases
MTX	Methotrexate
OD	Optical Densities
OHP	Oxaliplatin
PARP	Poly-(ADP-ribose) Polymerase
PBS	Phosphate-Buffered Saline
PELF	Cisplatin, Epirubicin, Leucovorin and 5-Flourouracil
Pt	Platinum
PVDF	Polyvinylidene Difluoride

RNA	Ribonucleic Acid
RPMI	Roswell Park Memorial Institute medium
SCID	Severe Combined Immunodeficiency
SDS-PAGE	Sodium dodecyl Sulfate-polyacrylamide Gel Electrophoresis
SRB	Sulforhodamine B
TCA	Trichloroacetic acid
TNF-α	Tumor Necrosis Factor-alpha
TNM	Tumour-Node-Metastases
TS	Thymidylate Synthase
VEGF	Vascular Endothelial Growth Factor
WT	Wild-Type

Abstract

Gastric cancer stands as the second most common cause of cancer death worldwide. Despite the increasing knowledge about the model of gastric tumourigenesis, the clinical management of gastric cancer patients has not improved significantly. The development of new approaches to overcome intrinsic and acquired resistance of gastric tumour cells, to currently available therapeutic options, constitutes a major challenge for gastric cancer research.

Recent studies suggest that a small group of cells, with the ability to initiate, progress and metastasize tumours are responsible for the resistance to current therapies in gastric cancer. This subpopulations of cancer cells was named cancer stem cells (CSCs), due to their ability to self-renew and to differentiate, specific characteristics for the stem cells. Accumulation of genetic and epigenetic alterations in putative gastric stem cells or their early progenies may trigger their malignant transformation into CSCs. The subpopulations of gastric CSCs are also believed to be relevant players in tumour chemoresistance due to the abnormal expression of specific proteins. However, remains, to be fully understood the biology of these CSCs, and their potential to be used as elective targets for therapeutic strategies in gastric cancer management.

The major question addressed in this work was whether gastric CSCs are involved in gastric cancer resistance to currently available drugs, and also trying to identify molecular mechanisms involved in chemoresistance of gastric CSCs. The adopted research strategy was the use of different gastric cancer cell lines, from which putative subpopulations of gastric CSCs were isolated, based on the expression of the CD44 surface marker. The isolated stem and non-stem subpopulations, as well as the WT population were exposed to drugs, currently used in gastric cancer chemotherapy,

namely cisplatin and 5-Fluorouracil (5-FU). From these cytotoxicity assays, the GI50 value was determined for each drug in each cell lines and subpopulations. Characterization of some proteins involved in apoptotic cascades was also performed in stem and non-stem subpopulations, in order to clarify the potential involvement of these mechanisms in a chemoresistance phenotype.

The obtained results showed the presence of potential CSCs in both cell lines in study (AGS and MKN45) expressing CD44, and these cell lines presented a significant difference in the resistance to cisplatin. Interestingly, the cell line with the lower percentage of putative CSCs, the AGS, was the one that presented a higher resistance to cisplatin. Also, the subpopulation enriched in gastric CSCs ($CD44^+$) did not show a significantly higher resistance to the tested drugs. The involvement of an overexpression of anti-apoptotic or an underexpression of pro-apoptotic proteins couldn't be also correlated to the differences in the chemoresistance profiles between AGS WT, $CD44^+$ and $CD44^-$ subpopulations, among each other.

Considering all the obtained results, further studies are necessary in order to consider CD44 as a robust marker of gastric CSCs and the involvement of this subpopulation in gastric cancer resistance to chemotherapy also needs an extensive evaluation.

Keywords: Gastric cancer, cancer stem cells, 5-FU, cisplatin, CD44, cell sorting, apoptotic proteins.

Resumo

O cancro gástrico continua a ser o segundo tipo de cancro mais mortal em todo o mundo. Apesar do crescente conhecimento sobre a tumorigénese gástrica o tratamento de doentes com cancro gástrico não melhorou significativamente. O desenvolvimento de novas abordagens para ultrapassar a resistência intrínseca e as resistência adquirida pelas células tumorais gástricas às opções terapêuticas actualmente disponíveis, constitui o maior desafio na investigação do cancro gástrico.

Estudos recentes sugerem que, a resistência às terapias actualmente utilizadas no tratamento do cancro gástrico é consequência de um número reduzido de células, com capacidade de iniciação, progressão e metastização de tumores. Esta subpopulação celular formada por células estaminais tumorais (CSCs), assim chamadas devido à sua capacidade de auto-regeneração e diferenciação, características que são idênticas às das células estaminais. A acumulação de alterações genéticas e epigenéticas em células estaminais ou em células directamente delas derivadas pode levar a uma transformação maligna, originando CSCs. Além disso, pensa-se que subpopulações de CSCs desempenham um papel relevante na resistência do cancro à quimioterapia devido a uma expressão anormal de proteínas específicas. Contundo, ainda está por esclarecer a biologia destas células e o seu potencial como alvos nas estratégias terapêuticas do cancro gástrico.

O principal objectivo deste trabalho foi avaliar se as subpopulações de células estaminais tumorais gástricas estão envolvidas na resistência do cancro gástrico aos fármacos utilizados actualmente na quimioterapia, e também, tentar identificar mecanismos moleculares que pudessem estar envolvidos na quimio-resistência das células estaminais tumorais gástricas. Para tal, a estratégia adoptada consistiu na utilização de diferentes linhas celulares gástricas, a partir das quais se isolaram as

potenciais subpopulações de CSCs, com base na expressão do marcador membranar CD44. As duas subpopulações obtidas, assim como a população WT foram expostas a agentes quimioterapêuticos usados regularmente no tratamento do cancro gástrico, nomeadamente a cisplatina e 5-Fluoruracil (5-FU). A partir de ensaios de citotoxicidade, o valor de GI50 foi determinado para cada droga em cada linha e em cada subpopulação. Neste trabalho foi ainda efectuada uma caracterização de proteínas envolvidas em cascatas apoptóticas nas linhas parentais, assim como nas subpopulações, com o objectivo de clarificar o potencial envolvimento deste mecanismo no fenótipo quimio-resistente destas células.

Os resultados obtidos demonstraram a presença de potenciais CSCs em ambas as linhas celulares estudadas (AGS e MKN45. As linhas apresentaram níveis diferentes de resistência à cisplatina. Sendo, a linha com menor percentagem de CSCs ($CD44^+$), a AGS, a que apresentou uma maior resistência à cisplatina. Verificou-se, também, que a subpopulação enriquecida em CSCs gástricas não apresentava uma maior resistência às drogas testadas. O envolvimento de uma sobre-expressão de proteínas anti-apoptóticas ou uma sub-expressão de proteínas pró-apoptóticas também não pôde ser correlacionado com os perfis de quimio-resistência da linha AGS WT e das subpopulações $CD44^+$ e $CD44^-$.

Considerando os resultados obtidos, tornam-se necessários futuros estudos complementares, de modo a provar que o CD44 é um bom marcador de CSCs gástricas, e que esta subpopulação está envolvida na resistência do cancro gástrico à quimioterapia.

Palavras-chave: Cancro gástrico, Células estaminais tumorais, 5-FU, cisplatina, separação celular, proteínas apoptóticas.

1. Introduction

1.1. Cancer epidemiology

Cancer remains a worldwide major public health problem. Despite consistent development medical research, the global burden of cancer has more than doubled during the last 30 years, being today a major cause of death. In the past, cancer was generally considered to be a disease of high-resource and industrialised western countries. However, nowadays, the situation has changed dramatically, and the majority of cancer cases are most common in low- and medium-resource countries. In the year of 2008, cancer was responsible for 7.6 million deaths (about 13% of all deaths worldwide), with 56% of new cancer cases and 63% of the cancer deaths occurring in the less developed regions of the world. The International Agency for Research on Cancer (IARC) estimated, for the same year, that cancer affected 28 million persons (within 5 years from initial diagnosis) and over than 12 million new cases were diagnosed (Accardi, 2008). The most commonly diagnosed cancers worldwide are lung (1.61 million cases, corresponding to 12.7% of the total), breast (1.38 million, 10.9% of the total) and colorectal cancers (1.23 million, 9.7% of the total). The most abundant causes of cancer related deaths are lung cancer (1.38 million, 18.2% of the total), stomach cancer (738 000 deaths, 9.7% of the total) and liver cancer (696 000 deaths, 9.2% of the total) (Ferlay et al., 2010a). The continued growth and ageing of the world's population will significantly affect the cancer burden. It could be expected that, by the year of 2030, 27 million new cases of cancer and 17 million cancer related deaths will have occurred (Accardi, 2008).

In Europe alone, cancer was responsible for 1.72 million deaths, 56% of them in men and 44% in women in the year of 2008. In the same year, about 3.2 million new cases, 53% of them in men and 47% in women have been diagnosed. The most common

cancers were colorectal (13.6% of the total), followed by breast (13.1%), lung (12.2%) and prostate cancer (11.9%). The major cause of cancer related deaths in Europe during 2008 was lung cancer accounting for one fifth of the total number of cancer related deaths. The second major cause of cancer death was colorectal cancer (12.3%), followed by breast cancer (7.5%) and by stomach cancer (6.8%) (Ferlay et al., 2010b). A study carried out by Pinheiro and colleagues (2003), the latest work performed in Portugal focusing on the incidence of cancer, where they estimated the cancer incidence in Portugal during 1996 to 1998, predicted for the year 2000 a total of 19 880 new cancer cases among men, and 17 000 new cancer cases in women in Portugal.

Due to the high levels of incidence and worldwide mortality, cancer is one of the areas of research where enormous efforts are being developed in order to better understand this disease, and consequently, to discover more efficient treatments and/or diagnostic tests.

Over the past four decades, cancer research has generated a rich and complex set of knowledge, showing that cancer is a disease that involves dynamic changes in the genome. These changes include mutations and/or abnormal expression of oncogenes, tumour suppressor genes, translocations and other chromosomal alterations (Argyle and Blacking, 2008; Hanahan and Weinberg, 2000). Most proto-oncogenes are key genes involved in the control of cell proliferation and growth. Namely growth factors, growth factor receptors, protein kinases, signal transducers, nuclear proteins and transcription factors (Hanahan and Weinberg, 2000). A proto-oncogene is converted in an oncogene as a result of several events in the genetic material of the target cell. The activated allele results in a dominant gain of function, which means that only one allele has to be affected to acquire a phenotypic change. The mechanisms involved on the activation of oncogenes include: chromosomal translocation (where proto-oncogenes are translocated

within the genome), gene amplification (multiple gene copies), point mutations (single base changes in the DNA sequence) and insertions of viral sequences (Argyle and Blacking, 2008).

Changes in genes can lead to either a stimulatory or inhibitory effect on cell growth and proliferation. The stimulatory effects are provided by the proto-oncogenes, where mutations or translocations of these genes create positive signals leading to uncontrolled growth. On the other hand, tumour formation can be obtained by a result of loss of the inhibitory functions associated with the tumour suppressor genes. Mutations in these types of genes are recessive, in other words, a mutation in one gene copy usually has no effect, as long as a reasonable amount of wild-type (WT) protein remains (Argyle and Blacking, 2008).

Much of the research conducted in this area has shown that, despite the many potential causes of cancer and carcinogenic pathways, the transformation of a normal cell into a cancer cell requires very few molecular, biochemical and cellular changes, which have the ability to confer the malignant phenotype to the cancer cell. Despite the wide variety of cancers types, these changes seem to be common to all types of cancer (Argyle and Blacking, 2008).

These alterations, responsible for the malignant growth of the cells, are acquired during carcinogenesis and are the following ones: self sufficiency in growth signals, insensitivity to anti-growth signals, evasion of programmed cell death (apoptosis), limitless replicative potential, an ability to sustain angiogenesis, tissue invasion and metastasis and inflammatory microenvironment (Figure 1) (Colotta et al., 2009; Hanahan and Weinberg, 2000).

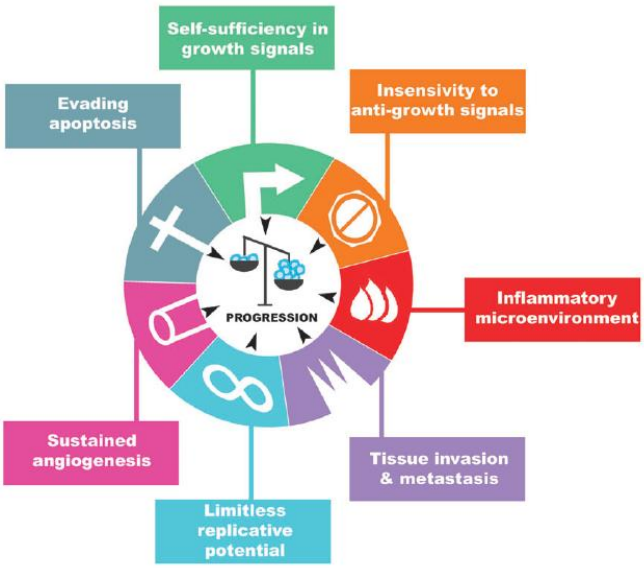

Figure 1: The different pathways to cancer. Despite being considered to be a complex disease, cancer can be defined by the acquisition of the depicted alterations responsible for the transformation of a normal cell into a cancer cell (Adapted from Colotta et al., 2009).

In a simple way, cancer can be considered as a breakdown in cellular homeostasis, which can lead to an uncontrolled cell division and proliferation and ultimately to a disease stage. The cancer formation is the phenotypic outcome of a series of changes that require a long period of time to develop. After an initial step created by a carcinogenic agent on a cell, a period of tumour promotion begins (Figure 2). The first step, known as initiation, is rapid and affects the genome of the cell, and, if the damage is not repaired by the cell, the promoting factors may lead the cell towards a malignant phenotype. Contrarily to the initial step, the progression can be a very slow process, and fortunately may not even manifest during the lifetime of the individual (Argyle and Blacking, 2008).

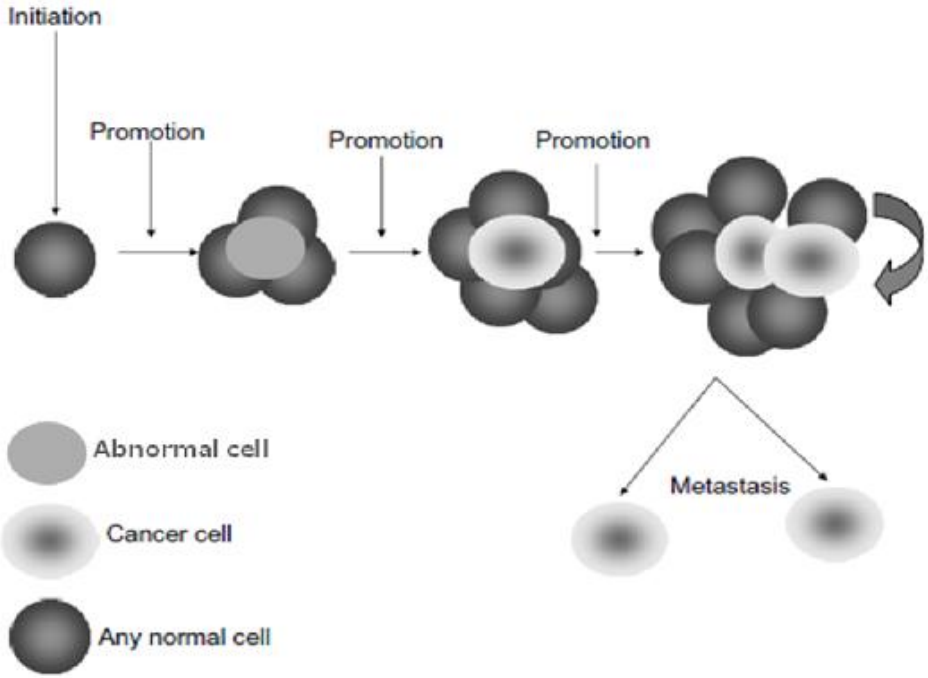

Figure 2: The model of carcinogenesis. Cancer formation is the result of a series of changes that may have taken a long period of time to develop. They can occur in any cell type in the body. Following an initiation step, a period of tumour promotion occurs. Each stage of multi-step carcinogenesis reflects genetic changes in the cell with a selection advantage that drives the progression towards a highly malignant cell (Adapted from Argyle and Blacking, 2008).

The formation of cancer is thought to be a combination of multiple genetic and environmental risk factors (Pharoah et al., 2004). It is important to take into account, that the common genetic alterations may not have, by themselves, substantial impact risk, but, when associated with exposure to environmental carcinogens could lead to tumour development (Bartsch and Hietanen, 1996). Epidemiological studies have estimated that up to 80 to 90% of all cancers are related to environmental factors, tobacco smoke, and diet (Doll and Peto, 1981). Tobacco smoking is unquestionably a

major human carcinogen factor, as about 30% of all cancer cases worldwide are caused by tobacco, particularly lung cancer. Modifiable risk factors for cancer have been identified, including alcohol consumption, excessive exposure to sunlight, lack of physical activity, overweight and obesity, dietary factors, occupational exposures and chronic infection (Accardi, 2008; Bartsch and Hietanen, 1996). Since most carcinogens require metabolic activation before binding to DNA (deoxyribonucleic acid), individual features of carcinogen metabolism play an essential role in the development of environmental cancer (Bartsch and Hietanen, 1996).

The knowledge about the involvement of these pathways in several tumour types is still scarce. So, it is extremely important to decode and understand the mechanisms involved in the development of such tumours. It is therefore important develop preventive measures, new diagnostic tests and more effective therapeutic approaches.

1.2. Gastric Cancer

Among the most frequent and less treatable cancers worldwide presently is the gastric cancer. According to the most recent data, gastric cancer is the fourth most common cancer worldwide, with 989 000 (7.8%) cases per year, and it is the second most common cause of cancer death worldwide (738 000 deaths, 9.7% of the total) (Ferlay al., 2010a). The geographical distribution of gastric cancer shows a wide geographical variation, with the highest incidence rate in China (about 42% of the all cases), Japan, Eastern Europe and certain countries of Latin America, being the low-risk populations seen in North America, India, the Philippines, most countries in Africa, some western European countries and Australia (Curado et al., 2008; Ferlay et al., 2010a; Kamangar et al., 2006; Parkin et al., 2005). Men have a higher risk to develop

gastric cancer than women (male-to-female ratio of 2:1), a consistent phenomenon in many geographic areas (Ferlay et al., 2010a; Takaishi et al., 2008).

In Europe, during the year of 2008, gastric cancer was the fourth cause of death from cancer related diseases, with 117 000 registered cases, about 6,8% of the total cases, and was the fifth most common cancer diagnosed (Figure 3) (Ferlay et al., 2010b).

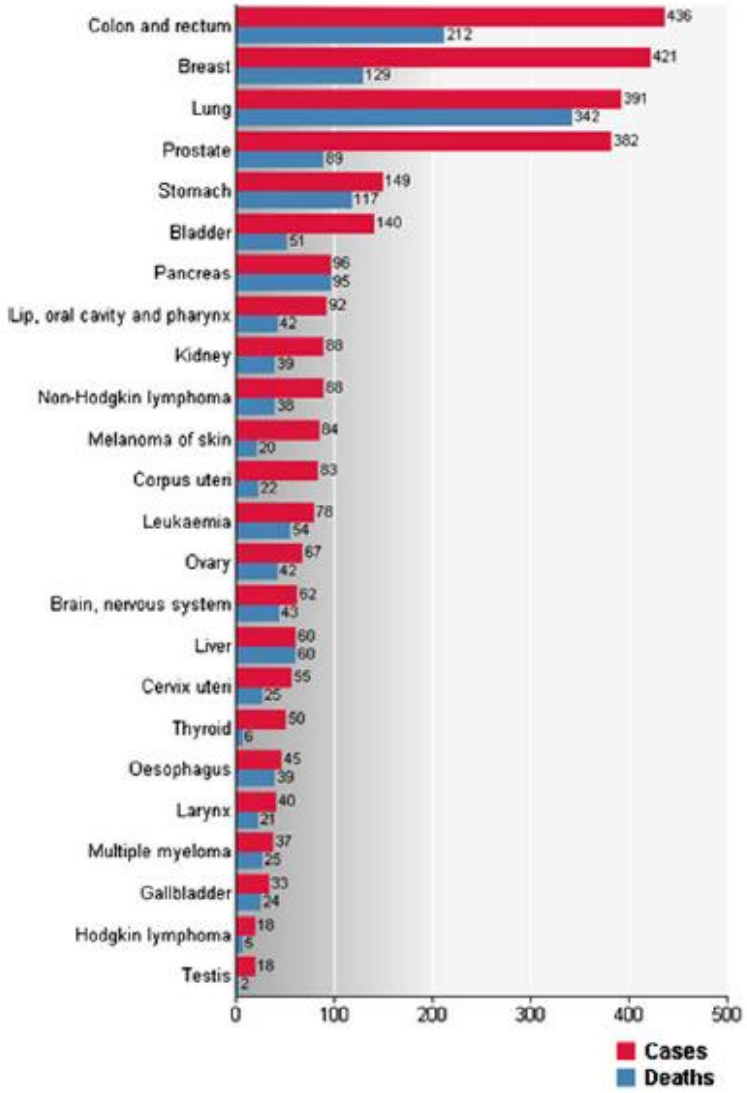

Figure 3: Estimated numbers of cancer cases and cancer deaths in Europe (in thousands) (Adapted from Ferlay et al., 2010b).

The vast majority of gastric cancers are adenocarcinomas (90%) and the remaining 10% are lymphomas or Gastrointestinal Stromal Tumours (GIST) (Tepes, 2009). Gastric adenocarcinomas are classified according to the pathological characteristics of the lesion and the origin site of the tumour. The histological classification mostly used is the Lauren's classification (1965), in which the adenocarcinomas are classified in, intestinal and diffuse. Intestinal-type carcinomas, with cohesive, glandular-like cell groups, usually occur at a late age, are more common in men and progress through a relatively well-defined series of pre-neoplastic histologic steps (Lauren, 1965; Peek and Blaser, 2002). The diffuse-type gastric adenocarcinoma, with infiltrating non-cohesive tumour cells, affects men and women nearly equally, is more common within younger people, and consists of individually infiltrating neoplastic cells that do not form glandular structures, are not associated with intestinal metaplasia and are poorly differentiated (Lauren, 1965; Takaishi et al., 2008). The intestinal-type gastric cancer, arises through sequential stages of chronic gastritis and develops through the intermediate stages of atrophic gastritis, intestinal metaplasia, dysplasia, to finally reach the gastric cancer stage, in a process known as the Correa pathway (Correa, 1995; Correa and Houghton, 2007).

Gastric carcinogenesis is a long-term process that may take several decades until a gastric cancer stage is reached. As indicated by the Correa pathway, the progression has several intermediate stages, and the precursor lesions can be associated to risk factors. For instance, epidemiological evidences have shown that *Helicobacter pylori* (*H. pylori*) is the primary risk factor for gastric cancer, being responsible for at least two thirds of the cases (Takaishi et al., 2008). *Helicobacter pylori*, a spiral shaped, microaerophilic and gram-negative bacterium that colonises the human stomach, was first isolated in 1984 by Marshall and Warren. They demonstrated the role of this

bacterium in gastritis and peptic ulcer disease. In 1994, the IARC classified *H. pylori* as carcinogenic to humans, based on epidemiological evidence for the correlation between this bacterium and gastric cancer and gastric mucosa-associated lymphoid tissue (MALT) lymphoma.

The infection by *H. pylori* is one of the most common chronic bacterial infections in humans with an estimated prevalence of 50% worldwide and 90% in developing countries (Accardi, 2008). In high-prevalence populations, the infection is frequently acquired in childhood before the age of ten and persists throughout life (Accardi, 2008; Tepes, 2009). Fortunately, the prevalence of *H. pylori* infection is declining in many developed countries and a later acquisition of this pathogen may also contribute to low infection prevalence in children and young adults.

Several studies indicate that gastric cancer is also associated with a high-salt diet, a low intake of fruits and vegetables, and smoking (Ishikawa et al., 2005; Ushijima and Sasako, 2004). Smokers have a 50–60% increased risk of stomach cancer, as compared to non-smokers. Additionally, smoking is responsible for approximately 10% of all cases (IARC, 2004), and the recent decline in smoking prevalence in men from developed countries may account for part of the fall in gastric cancer rates (Ladeiras-Lopes et al., 2004). The two major changes that could be made at a population level to reduce gastric cancer incidence are improvement in diet (rich in fruit and vegetables and lower in salt) and reduction in the prevalence of *H. pylori*.

The epidemiology of gastric cancer indicates that its risk is dominated by environmental causes. However, there is still a role played by genetic factors, including the blood type (the frequency of gastric cancer is higher in individuals with blood group A) and germline mutations in the gene *CDH1* (Cadherin 1), which encodes for the cell

adhesion protein E-cadherin, like in familial diffuse gastric cancer (Guilford et al., 1998).

During the last decades there has been a decline in the incidence and mortality rates of gastric cancer worldwide (Accardi, 2008; Ferlay et al., 2010a). Nevertheless, the number of diagnosed cases and mortalities remained high, and is thus important to find new approaches capable to improve the overall prognosis of gastric cancer.

The survival rate of patients with gastric cancer is substantially worse than that of patients with most other solid malignancies. This happens because the disease is asymptomatic in early stages and more than half of gastric carcinomas are diagnosed in advanced stages, when the metastases have already progressed and the resection is not possible (Wesolowski et al., 2009). In patients diagnosed in earlier stages of the disease, more than 50% undergo to surgery, and even after a curative resection, 60% eventually relapse locally or with distant metastases with a median survival time of 4–6 months, sometimes improved up to 7-10 months with chemotherapy (Neri et al., 2007).

The five-year relative survival rate in most regions is about 20% (in USA is 24% (Ries et al., 2007)), though it is up 60% in Japan due to the practice of extensive early screening and treatment (Kamangar et al., 2006). The complete resection (surgery) of the tumour is the only treatment that offers potential cure (Bozzetti et al., 1999; Cervantes et al., 2004).

When patients present advanced or metastatic disease, and tumours that cannot be resected, chemotherapy or radiotherapy are the best options to improve quality of life and prolong survival (Casaretto et al., 2006; Khushalani, 2008). Actually, chemotherapy can improve the median survival time up to one year (reviewed by Xu and Teng in 2009).

1.3. Chemotherapy in Gastric Cancer: cisplatin and 5-Fluorouracil with the leading role.

The therapeutic approach, in gastric cancer, is determined by the extent (stage) of the disease in the time of diagnosis. The stage of the tumour is determined using the TNM (tumour-node-metastases) system as depicted in Table I (Rindi et al., 2006). Patients in early stages (stages 0 and I) at the time of diagnosis have a better prognosis than patients in advanced stage (T3, T4 or node-metastatic). In patients with advanced disease, complete recessions (R0) are difficult, and there is a high risk of local recurrence and distant (metastasis). Even though, surgical resection remains the primary curative approach for gastric cancer (Cervantes et al., 2004), some studies have shown that surgery alone is less than satisfactory, with cure rates approaching only 40% (Hundahl et al., 2000).

The use of chemotherapy targeted (with or without radiotherapy) based upon the increased knowledge about cancer cells genetics and their behaviour holds perhaps the greatest potential to improve patients survival (Cervantes et al., 2004).

Chemotherapy for the treatment of gastric cancer began in the 1960s and 70s but despite 50 years of clinical research, there are many questions to be clarified. Most importantly, despite the toxicity and costs associated with treatment, chemotherapy plays a justified role in the treatment of patients with advanced disease, where quality of life may be a critical issue (Earle and Maroun, 1999; Kohne et al., 2000). Several trials demonstrated that the use of chemotherapy (before and after resection) is comprehensible, since its use provided improvements in response rates, progression-free survival and overall survival (Glimelius et al., 1997; Murad et al., 1993; Pyrhönen et al., 1995; Scheithauer et al., 1995). The timing of the initiation of chemotherapy relatively

to the diagnosis and progression was also shown to have an effect on the outcome. A treatment applied right after the diagnosis improved the quality of life and time of survival (10 months *versus* 4 months) when compared to a treatment started only at progression stages (Guilford et al., 1998).

Neoadjuvant chemotherapy has many advantages in the treatment of gastric cancer. For instance, it is believed that this kind of chemotherapy is effective in patients at advanced stages, because it can be responsible for tumour shrinkage, allowing the resection of the tumour. Moreover, patients with locally advanced gastric cancer can also have distant micrometastases, and if surgical strategy has been used first, the metastasis often aren't treated in time and could interfere with the post-operative treatments. Therefore, pre-operative chemotherapy can be helpful in avoiding this problem (Xu and Teng, 2009). In 2005, a trial named as MAGIC (medical research council adjuvant gastric infusional chemotherapy) trial, conducted by Cunningham and colleagues tested the efficiency of the pre-operative chemotherapy. For the study, the authors recruited 503 patients, who were divided in two groups, surgery alone and pre-operative treatment plus surgery. The treatment group received 3 courses of ECF (epirubicin, cisplatin and 5-fluorouracil in continuous infusion) before and after surgery and the results showed that the treatment group had a higher rate of T1 and T2 tumours (51.7% vs. 36.8%) than the surgery alone group in post-operative pathological tests. In addition, the 5-year survival rate of the treatment group was about 13% (36% vs. 23%) higher than the surgery alone group. Only 42 % of the patients in the treatment group completed post-operative ECF adjuvant chemotherapy. The authors concluded that the pre-operative chemotherapy is efficient, and it can decrease the diameter of the tumour and depth of invasion, as well as to improve lymph node status, and therefore manage

the effect of tumour downgrading. However, an improvement in complication and mortality rates of the treatment group was not observed (Cunningham et al., 2005).

Table I: TNM classification and stages of gastric tumours (Adapted from Rindi et al., 2006).

TNM			
T – primary tumour			
TX	Primary tumour cannot be assessed		
T0	No evidence of primary tumour		
Tis	In situ tumour/dysplasia (<0.5 mm)		
T1	Tumour invades lamina propria or submucosa and ≤1 cm		
T2	Tumour invades muscularis propria or subserosa or >1 cm		
T3	Tumour penetrates serosa		
T4	Tumour invades adjacent structures		
	For any T, add (m) for multiple tumours		
N – regional lymph nodes			
NX	Regional lymph nodes cannot be assessed		
N0	No regional lymph node metastasis		
N1	Regional lymph node metastasis		
M - distant metastasis			
MX	Distant metastases cannot be assessed		
M0	No distant metástases		
M1[a]	Distant metastasis		
Stage			
Disease stages			
Stage 0	Tis	N0	M0
Stage I	T1	N0	M0
Stage IIa	T2	N0	M0
IIb	T3	N0	M0
Stage IIIa	T4	N0	M0
IIIb	Any T	N1	M0
Stage IV	Any T	Any N	M1

[a]M1 specific sites defined according to Sobin and Wittekind (2002).

Since 1960, a variety of chemotherapeutic drugs, to the treatment of gastric cancer have been developed, such as 5-fluorouracil (5-FU), cisplatin (cis-Diamminedichloroplatinum) (CDDP), methotrexate (MTX), doxorubicin (ADM), etoposide, leucovorin (LV) and mitomycin C (MMC). The use of 5-FU began in the 1960s, and during the following decades several combinations based on 5-FU were developed, such as, FAM (5-FU, adriamycin and MMC), ELF (etoposide, LV and 5-FU) and FAMTX (5-FU, adriamycin and MTX). During the 1980s and 1990s, several drug treatments incorporating CDDP were also developed, such as EAP (etoposide,

adriamycin and CDDP), FLEP (5-FU, LV, epirubicin and CDDP), CF (CDDP and 5-FU in continuous infusion) and PELF (CDDP, epirubicin, LV and 5-FU). In the years of 2000 others pharmacological combinations were used, such as ECF (epirubicin, CDDP and 5-FU in continuous infusion) (Cervantes et al. 2004; Rivera et al.2007) (Figure 4).

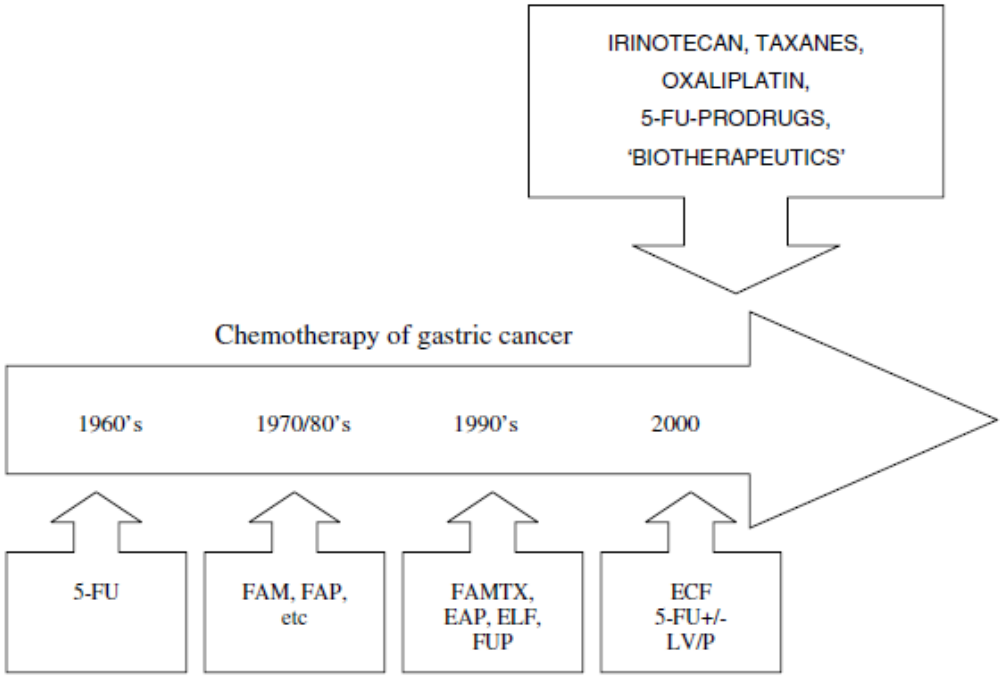

Figure 4: An overview of the history of chemotherapy in the treatment of gastric cancer. 5-FU (5-fluorouracil), LV (leucovorin), FAM (5-FU, doxorubicin and mitomycin C), FAP (5-FU, doxorubicin and cisplatin), FAMTX (5-FU doxorubicin and methotrexate), EAP (etoposide, doxorubicin and cisplatin) ELF (etoposide, LV and 5-FU), FUP (5-FU and cisplatin), ECF (epirubicin, cisplatin and 5-FU) and P (cisplatin) (Adapted from Cervantes et al. 2004).

Nowadays, it is well recognised that a combination of chemotherapy regimens improve patient outcomes, but there is no consensus standard. 5-FU given as a continuous infusion in combination with CDDP has consistently demonstrated

superiority in terms of response rate and time to disease progression compared with 5-FU monotherapy and other combination regimens, although it has not shown a significant survival advantage (Kim et al., 1993; Ohtsu et al. 2003; Vanhoefer et al., 2000). As a result of these findings, CF combination was subsequently adopted as a standard reference regimen in Korea and many other countries. In Europe, triple combination regimens have been widely tested, and ECF is accepted as a standard regimen in the UK and parts of Europe (Kang et al., 2009). 5-FU plus CDDP in patients with gastric cancer demonstrated a favourable outcome, with response rates of 43% to 45% (Kondo et al., 1996; Ohtsu et al., 1994; Rougier et al., 1994). Even though there is no global consensus on a standard regimen for gastric cancer, a combination of 5-FU plus CDDP is the most commonly used treatment worldwide (Hironaka et al., 2010).

Presently, 5-FU is the most common drug used for gastric cancer both in adjuvant and advanced settings (reviewed by Shimoyama in 2009). An analogue of uracil, 5-FU, is incorporated intracellularly into the same DNA and RNA (ribonucleic acid) metabolic pathways as uracil. This incorporation of 5-FU is the essential mechanism of its cytotoxic effect. There are substantial numbers of enzymes involving 5-FU incorporation into DNA and RNA synthesis pathways (anabolic and catabolic pathways of 5-FU). 5-FU is converted into several active metabolites which disrupt RNA synthesis and inhibit the action of thymidylate synthase (TS), a key enzyme of DNA synthesis. On the other hand, 5-FU is catabolized by the rate-limiting enzyme dihydropyrimidine dehydrogenase (DPD) (Huang and Ratain, 2009; Park et al., 2004; Shimoyama, 2009).

More than 80% of administered 5-FU is degraded primarily in the liver (Diasio and Harris, 1989; Heggie et al., 1987) where DPD is abundantly expressed, to its inactive metabolite dihydrofluorouracil (DHFU), and the remaining portion of 5-FU is

incorporated into DNA and RNA synthesis pathways and exerts its cytotoxic effect via the formation of three main active metabolites: fluorouridine monophosphate (FUMP), fluorodeoxyuridine triphosphate (FdUTP), and fluorodeoxyuridine monophosphate (FdUMP). These metabolites disrupt RNA synthesis and the action of TS (Park et al., 2004; Shimoyama, 2009).

Several trials (in vitro and in vivo) demonstrated that CDDP enhances the anti-tumour activity of 5-FU (Esaki et al., 1992; Shirasaka et al., 1993). The anti-tumour activity of CDDP is generally attributed to its formation of DNA adducts, both intrastrand and interstrand crosslinks, which induce structural distortions in DNA (Trimmer and Essigmann, 1999; van Moorsel et al., 1999). The main adduct is formed when the platinum (Pt) is bound to two adjacent guanines (Pt-GG); another major intrastrand crosslink is formed when Pt is bound to adenine and an adjacent guanine (Pt-AG). An additional intrastrand crosslink is formed when Pt is bound to two guanines separated by one or more other nucleotide(s) (G-Pt-G) and the interstrand crosslinks on two guanines on opposite strands. The DNA adducts of CDDP are thought to mediate its cytotoxic effects by inhibiting DNA replication and transcription and, ultimately, by inducing programmed cell death, or apoptosis (Trimmer and Essigmann, 1999).

In the last decades, several trials have been conducted, using new chemotherapy drugs, such as, docetaxel, irinotecan, oxaliplatin, paclitaxel, oral fluoropyrimidines - capecitabine, S1, among others. In table II, are shown some of the trials conducted by several authors, where many, but not only, combinations with CDDP and 5-FU are tested. The outcome of the trials is also demonstrated in the form of the response rate and median survival time of the patients.

Targeting drugs is one of the hot topics in cancer research in recent years. Given limited effectiveness of chemotherapeutic drugs for gastric cancer, many researchers

search for combination regimes containing targeting drugs and hope for a better efficiency. Currently, many targeting drugs have been applied in studies of gastric cancer, including drugs to target tyrosine kinase inhibitor Gefitinib (Iressa) and Erlotinib (Tarceva), anti-vascular endothelial growth factor (VEGF) receptor monoclonal antibody Bevacizumab (Avastin) and anti-HER-2 monoclonal antibody Trastuzumab (Herceptin) (Xu and Teng, 2009).

The efficiency of cancer therapies as chemotherapy and radiotherapy is often compromised by intrinsic or acquired resistance to the tumour cytotoxic agents and ionizing radiation (Schatton et al., 2009), which is believed to be an important cause of low rates of successful treatment of gastric cancer.

In many cases, after chemotherapy the tumour is significantly affected, but sometimes there is a reappearance and redevelopment, caused by tumour cells displaying multi-resistance to the used drugs. In a conventional view, tumour cells acquire resistance due to a number of mechanisms such as mutations or underexpression of the targets of the cytotoxic agents and inactivation or elimination of drugs by the cell (Dean et al., 2005). However, these molecular changes do not contemplate the physiological and morphological heterogeneity of the tumour cells (Hanahan and Weinberg, 2000).

Recent evidence suggests that the large heterogeneity of the tumour, as well as the significant resistance of tumour cells towards chemotherapeutic agents, is due to the existence of a cell subpopulation responsible for the initiation, progression and tumour recurrence: the cancer stem cells (CSCs).

Table II: A general overview on a broad range of trials comparing various regimens of chemotherapeutic treatments used in gastric cancer.

Trial	*N*	*Treatment Regimen*	*Response Rate (%)*	*Median Survival Time (Mouths)*
Ajani et al. (1993)	25	EFP	80	15
Al-Batran et al. (2004)	41	OHP + 5-FU	43	9.6
Al-Batran et al. (2008)	108	5-FU + LV + CDDP	25	8.8
	112	5-FU + LV + OHP	34	10.7
Asserson et al. (2004)	40	Irinotecan + 5-FU + folinic acid	-	7.3
Cho et al. (2006)	19	Paclitaxel + 5-FU + folinic acid	7.1	7
Cullinan et al. (1985)	51	5-FU	18	7
	51	FAM	38	7
Cullinan et al. (1994)	69	5-FU	-	6.1
	53	FAMe	-	6.1
Cunningham et al. (2008)	263	ECF	41	9.9
	245	EOF	42	9.3
	250	ECX	46	9.9
	244	EOX	48	11.2
Giordano et al. (2006)	44	Doc + capecitabine	-	9.4
Giuliani et al. (2005)	38	Irinotecan + MMC	3	8
Jeong et al. (2008)	52	OHP + 5-FU + folinic acid	-	6.6
Jeung et al.(2007)	57	S-1	6	8.3
Kang et al. (2006)	139	Cape + CDDP	41	10.5
	137	5-FU + CDDP	29	9.3
Kelsen et al. (1996)	56	FAMTX + CDDP + 5-FU	50	15
Kim et al. (1993)	94	5-FU	26	7.5
	98	FAM	25	7
	103	5-FU + CDDP	51	9.2
Lee et al. (2007)	48	OHP + 5-FU + LV	66	14.8
Leichman et al. (1992)	38	CDDP + 5-FU	68	>17
Nguyen et al. (2006)	50	EPI + Doc	15.5	5
Ott et al. (2003)	49	CDDP + folinic acid + 5-FU	71	25.4
Park et al. (2006)	20	OHP + Cape	65	-
Persiani et al. (2008)	24	EEP	42	40
Schuhmacher et al. (2001)	42	EAP	66	19
Shin et al. (2008)	45	Cape + Doxorubicin	-	29.1
Thuss-Patience et al. (2005)	45	EPI + CDDP + 5-FU	37	9.5
	45	Doc + 5-FU	35	9.7
Vanhoefer et al. (2000)	85	FAMTX	12	6.7
	81	5-FU + CDDP	20	7.2
Webb et al. (1997)	126	ECF	46	8.7
	130	FAMTX	21	6.1
Wils et al. (1991)	103	FAM	9	7.2
	105	FAMTX	41	10.5
Yoshikawa et al. (2009)	55	Irinotecan + CDDP	55	14.6
Zhong et al. (2008)	48	Doc + OHP	-	8

Abbreviations: 5-FU (5-flourouracil), Cape (capecitabine), CDDP (cisplatin), Doc (docetaxel), EPI (epirubicin), LV (leucovorin), MMC (mitomycin C), OHP (oxaliplatin), EAP (etoposide+adriamycin+CDDP), ECF (EPI+CDDP+5-FU), ECX (EPI+CDDP+Cape), EEP (epidoxorubicin+etoposide+CDDP), EFP (etoposide+5-FU+CDDP), EOF (EPI+OHP+5-FU), EOX (EPI+OHP+Cape), FAM (5-FU+adriamycin+MMC), FAMe (5-FU+adriamycin+methyl lomustine), FAMTX (5-FU+adriamycin+methotrexate).

1.4. Cancer Stem Cells

Organs are composed by a multitude of differentiated cells that perform diverse functions. An underlying homeostatic system exists to replace senescent differentiated cells and tissue loss following injury. This hierarchical system typically involves several stages that have decreasing proliferation capacity and simultaneous increasing commitment to differentiation (Miller et al., 2005). The most primordial cell in the hierarchy is the stem cell, a primitive cell that is defined by three main properties: a) differentiation that consists in the ability to give rise to a heterogeneous population of cells arranged in a hierarchical manner, includes various tissue-specific lineages, thereby building up the requisite critical mass toward replenishing the tissue of short-lived, differentiated cells; b) capacity of self-renewal, in which the stem cells are capable to undergo division and form new cells with the same potential of the mother cell, in other to maintain the stem cell pool; c) the homeostatic control, in which they have the ability to regulate and balance differentiation and self-renewal in the tissue or organ, according to environmental stimuli and genetic constraints (Bapat et al., 2009; Dalerba et al., 2007a; Lobo et al., 2007).

The self-renewal capability is sustained by symmetric and asymmetric cell divisions. In symmetric cell divisions, both daughter cells retain the same phenotype. On the other hand, in asymmetric cell divisions, each stem cell divides to generate one daughter with a stem-cell fate (self-renewal) and one daughter that differentiates (Hill and Perris, 2007; Morrison and Kimble, 2006) (Figure 5). Regulatory aspects are needed to maintain the normal behavior and functions of stem cells. They include maintenance of the stem cell in a quiescent, infrequently cycling, undifferentiated state, decisions to replicate and in what fashion (symmetrically or asymmetrically) and decisions to differentiate and along what lineage. These aspects of the stem cell

behavior may be intrinsic (within the programming of the stem cell itself), extrinsic (a response to external stimuli generated by the niche or imported soluble factors) or, most likely, both (Miller et al., 2005; Watt and Hogan, 2000).

Self-renewal is crucial to stem cell function, because it is required by many types of stem cells to persist for the lifetime of the animal. And this property is responsible for the enormous proliferative capacity of stem cells as well as the maintenance of the adult tissue (Al-Hajj and Clarke, 2004). Because cancer can be considered a disease of unregulated self-renewal, is important to understand the regulation of normal stem cell self-renewal as well as the regulation of cancer cell proliferation (Reya et al., 2001).

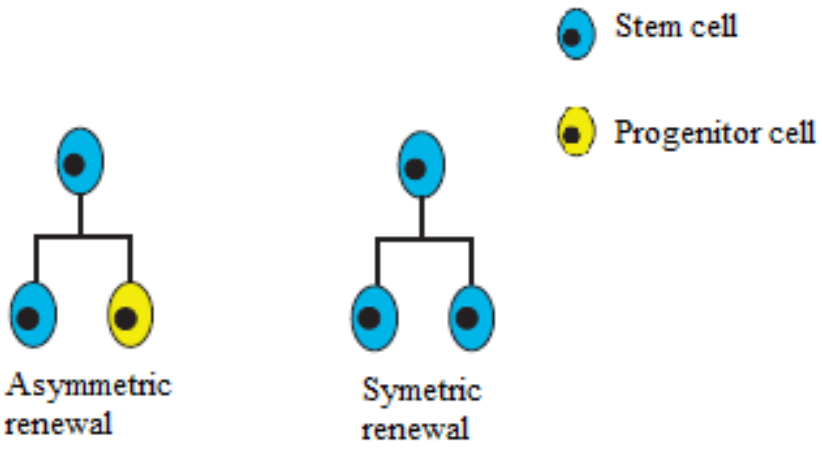

Figure 5: Stem cell division modes: symmetric self-renewal division results in two daughter stem cells, asymmetric self-renewal division results in one stem cell and one progenitor cell (adapted from Ashkenazi et al., 2008).

Despite the multitude of regulatory systems that prevent abnormal proliferation, mutations that result in aberrant mitoses do occur. Most mutations are inconsequential because the abnormal cell is eventually eliminated from the pool of replicating cells, but at some low frequency these mutations accumulate and may lead to cancer. Most of the

mutations leading to cancer affect cellular machinery that controls cell division, DNA damage, and signal transduction pathways. Stem cells may be preferential targets of initial oncogenic mutations because in most tissues in which cancer originates they are the only long-lived populations and are therefore exposed to more genotoxic stresses than their shorter-lived, differentiated progeny (Pardal et al., 2003; Reya et al., 2001). In fact, many studies have already demonstrated that cancers, particularly those of continually renewing tissues (blood, gut, skin), are in fact a disease of stem cells (reviewed by Alison et al., 2006). For more than a century it has been accepted that the cancer is constituted by a heterogeneous cell population (Fearon and Vogelstein, 1990; Nowell, 1976; Weiss, 2000).

Thus, having into account all the properties of the stem cells that were stated above, such as the similarities between these cells and the cancer cells in the self-renewal regulation and that the stem cells are the only ones that persist in the tissues for a sufficient length of time to acquire the requisite number of genetic changes for malignant development (Alison et al., 2006; Reya et al., 2001), as well as based on studies carried out both in vitro and in vivo that demonstrated that only a small fraction of cells within a tumour possess self-renewal capacity and are able to regenerate and sustain the growth tumour the cancer stem cell theory was formulated (Harrington, 2004; Tang et al., 2007; Vormoor et al., 1994).

In the XIX century, Rudolf Virchow, often thought as the father of modern cellular pathology, was the first to say that cancer is a disease that begins from an immature cell. This important work seeded the scientific landscape with the concept of cellular hierarchy, a tenet paramount to the cancer stem cell hypothesis (Lobo et al., 2007). The cancer stem cell model of tumour development and progression states that tumours, like normal adult tissues, contain a subset of cells that are self-renewable and

generate the differentiated progeny that comprise the tumour (Bonet and Dick, 1997; Clarke et al., 2006; Reya et al., 2001). These cells have been termed cancer stem cells to reflect their 'stem-like' properties and ability to continually sustain tumourigenesis. CSCs share important properties with normal tissue stem cells, including self-renewal (by symmetric and asymmetric division) and differentiation capacity. (Visvader and Lindeman, 2008).

The identification of the CSCs has been based in functional studies as well as in the expression of several markers. The first evidences of the existence of CSCs were reported by the group of John Dick (Bonnet and Dick, 1997; Lapidot et al., 1994), who identified these types of cells in acute myeloid leukemia (AML). In this study, they were able to isolate cells with a $CD34^{+}$ $CD38^{-}$ phenotype, markers for hematopoietic stem cells. These cells were then injected in immunodeficient mouse (SCID - Severe Combined Immunodeficiency) as a model to study the proliferation and self-renewal potential of transplanted human AML cells. The results showed that the animals maintain the AML phenotype (Dick, 1996), confirming the property of self-renewal. For the first time it was demonstrated that there are cells within the tumour which have properties similar to stem cells, *i.e.* the capacity to reconstitute the tumour when transplanted into an appropriate recipient (differentiation) through several rounds of transplantation (self-renewal). Another approach used to identify CSCs besides the implementation of these types of cells subcutaneously into SCID mice, is an *in vitro* method termed "spheroid colony formation" that involves culturing candidate CSCs under nonadherent conditions with serum-free media containing only EGF (epidermal growth factor) and bFGF (basic fibroblast growth factor) (Takaishi et al., 2008).

These evidences questioned a model of cancer development that is considered for many years. This model is known as the stochastic model, and postulated that all

cells in the tumour have the same proliferative capacity and are responsible for the initiation of the tumour in the same way (Figure 6) (Argyle and Blacking, 2008; Miller et al., 2005; Tang et al., 2007).

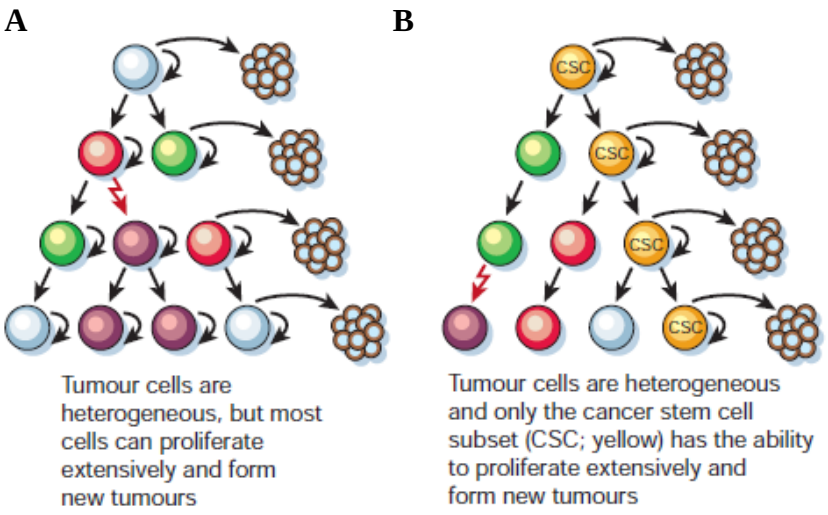

Figure 6: Two general models of heterogeneity in solid cancer cells. (**A**) Cancer cells of many different phenotypes have the potential to proliferate extensively, but any individual cell would have a low probability of exhibiting this potential in an assay of clonogenicity or tumourigenicity (stochastic model). (**B**) Most cancer cells have only limited proliferative potential, but a subset of cancer cells consistently proliferate extensively in clonogenic assays and can form new tumours on transplantation (Adapted from Reya et al., 2001)**.**

Several studies carried out in the last decade demonstrated that the CSCs may be originated in four ways. The first and the most accepted theory states that the CSCs are originated from stem cells subjected to transforming mutations. There is a transformation of stem cells residing in a tissue through the acquisition of abnormal growth and differentiation properties (Argyle and Blacking, 2008; Bapat et al., 2009; Beachy et al., 2004). The second possible origin of CSCs supports that these cells are

originated from progenitor cells targeted by mutations, and subsequent transformation of a local pool of early progenitors that require self-renewal properties (Bapat et al., 2009; Cozzio et al., 2003; Huntly et al., 2004; Lavau et al., 1997). The third hypothesis defends that there is a de-differentiation of committed progenitors or differentiated cells, in which a series of effective mutations that render committed transient-amplifying progenitor or differentiated somatic cells within a tissue immortal occur (Bapat et al., 2009; Okita et al., 2007). And finally, the last possible origin of CSCs is the fusion of tissue-specific stem cells with circulating bone marrow stem cells (Bapat et al., 2009; Houghton et al., 2004).

The first studies that suggested the existence of the theory of the CSCs were developed in leukemia models, although in the last few years, rapidly emerging investigations occur in numerous solid tumours. The first study in solid malignancy was carried out by Al-Hajj in 2003, where they identified and isolated CSCs in breast cancer. They described a $CD44^{+}CD24^{-}$ cell population that was significantly enriched for tumour-initiating capacity. Ever since, the identification and isolation of CSCs has been done in other solid tumours such as, gliomas (Sanai et al., 2005; Singh et al., 2003; Singh et al., 2004), melanoma (Fang et al., 2005), prostate cancer (Collins et al., 2005), osteosarcoma (Gibbs et al., 2005), colon cancer (Dalerba et al., 2007b), ovary cancer (Bapat et al., 2005) and head and neck squamous cell carcinoma (Prince et al., 2007).

The presence of gastric CSCs were first (and only) reported by Takaishi and colleagues in 2009. In this study they analyzed gastric cancer cell lines with defined surface markers and have identified the existence of gastric cancer initiating cells in the $CD44^{+}$ population. The CD44 positive gastric cancer cells showed the properties of self-renewal and the ability to produce differentiated progeny, consistent with the CSCs phenotype. The CD44 is a broadly distributed transmembrane glycoprotein that plays a

critical role in a variety of cellular behaviors, including adhesion, migration, invasion, and survival, and is considered as a characteristic marker of gastric CSCs. In order to prove that the $CD44^+$ population is constituted by gastric CSCs, the authors observed the. ‘‘spheroid colony formation’’ of gastric cancer stem cell and, also, transplanted FACS-sorted $CD44^+$ gastric cancer cells into the skin and stomach of SCID mice. They demonstrated the self-renewal properties of $CD44^+$ gastric cancer cells, by serial transplantation (isolating these cells from one xenograft and transplanting them into the skin and stomach of the second group of SCID recipient mice). Besides, Takaishi and co-workers also tested the resistance of both $CD44^+$ and $CD44^-$ subpopulations towards anticancer chemotherapy drugs. The results showed that the $CD44^+$ cells demonstrated the ability to form spheroid colony under non-adherent conditions in serum-free media as well as the capacity to form xenograft tumours in the stomach and skin of SCID mice, in which the $CD44^+$ fraction (20-30 thousand cells per site injection) generated tumours in both the skin and stomach of SCID mice after 8-12 weeks, while the $CD44^-$ fraction (30-100 thousand cells per site) did not generate tumours even after a longer observation period of 16 weeks. They demonstrated that these cells had the ability to self-renew through a serial passage of the $CD44^+$ fraction. And the results also showed that the $CD44^+$-induced gastric cancer presented a much greater resistance to the anticancer drugs when compared with the negative fraction. All these results are consistent with the standard definition of CSCs.

Presently, CD44 appears to be the most useful marker for prospective purification of gastric CSCs and has been identified as a CSCs marker for a number of other solid tumours (Al-Hajj et al., 2003; Collins et al., 2005; Dalerba et al., 2007b; Li et al., 2007; Prince et al., 2007).

The CD44 was first described by Dalchau et al. (1980), as a molecule present on the surface of T-lymphocytes, granulocytes and cortical thymocytes. CD44 mediates cell–cell and cell–matrix interactions in a large part through its affinity for hyaluronan (HA), a glycosaminoglycan constituent of extracellular matrices, but also potentially through its affinity for other ligands such as osteopontin, collagens, and matrix metalloproteinases (MMPs) (Cichy and Puré, 2003; Helmut et al., 1998). This glycoprotein is encoded by a single gene, but multiple isoforms of CD44 are generated by alternative RNA splicing. The gene for CD44 contains 20 exons, 12 of which are expressed by the most common form of CD44, known as standard or hematopoietic CD44 (CD44s), however at least 20 structurally different isoforms have been identified (Cichy and Puré, 2003; Marhaba and Zöller, 2004; Montgomery et al., 2004). This molecule is also considered a determinant of metastatic and invasive behavior in different malignancies, such as lung carcinoma, malignant melanoma, leukemia, breast cancer, as well as gastrointestinal carcinomas (Abbaszadegan et al., 2008; Ghaffarzadehgan et al., 2008; Wang et al., 2006; Zavrides et al., 2005).

As referred above, the CSCs have the ability to self-renew and generate the diverse cells that comprise the tumour and are responsible for continually sustaining tumourigenesis. So, if these types of cells are responsible for growth and metastasis, the successful elimination of the cancer requires anticancer therapy that is able to affect and eliminate the CSCs population. However, such therapy has proven to be very difficult to find so far.

1.5. Cancer stem cells and their role in resistance to tumour therapies

Cancer treatment has traditionally been based on the implicit assumption that human cancer populations are homogeneous (Tang et al., 2007). The currently available drugs used in the treatment of cancer can shrink tumours and metastatic tumours by killing mainly tumour bulk populations with limited self-renewal and proliferative potential, although these effects are usually transient and often do not appreciably extend the life of patients. This happens because the resistant CSCs may remain viable after the treatment and reestablish tumour growth, leading to the relapse and neoplasia progression (Lippman, 2000; Reya et al., 2001; Schatton et al., 2009; Stockler et al., 2000) (Figure 7).

The current chemotherapeutic approaches may be ineffective because CSCs, like all stem cells, are relatively quiescent, that is, they proliferate very infrequently. Another cause for a unsuccessful therapy is the fact that most of the current therapies do not target the signaling pathways (Notch, PTEN, BMI-1, WNT and p53) that tightly regulate self-renewal, which appear to be either mutated or epigenetically deregulated (Boman et al., 2008; Fan and Eberhart, 2008). Moreover, stem cells tend to be more resistant to chemotherapeutic agents and radiation than mature cell types from the same tissue (Borman et al., 2008; Jordan and Guzman, 2004). This resistance can be due to the presence of enhanced multidrug resistance, a higher expression of anti-apoptotic proteins, increased activity and expression of ABC (ATP-binding cassette) transporters and improved DNA repair mechanisms. Since this appears to hold true for CSCs, they would also display higher resistance to chemotherapeutics (Dean et al., 2005; Eyler and Rich, 2008; Reya et al., 2001). Other factors contributing for CSCs to acquire resistance

towards chemotherapy is the mutation or overexpression of the drug target, and inactivation or elimination of the drug from the cell (Dean et al., 2005).

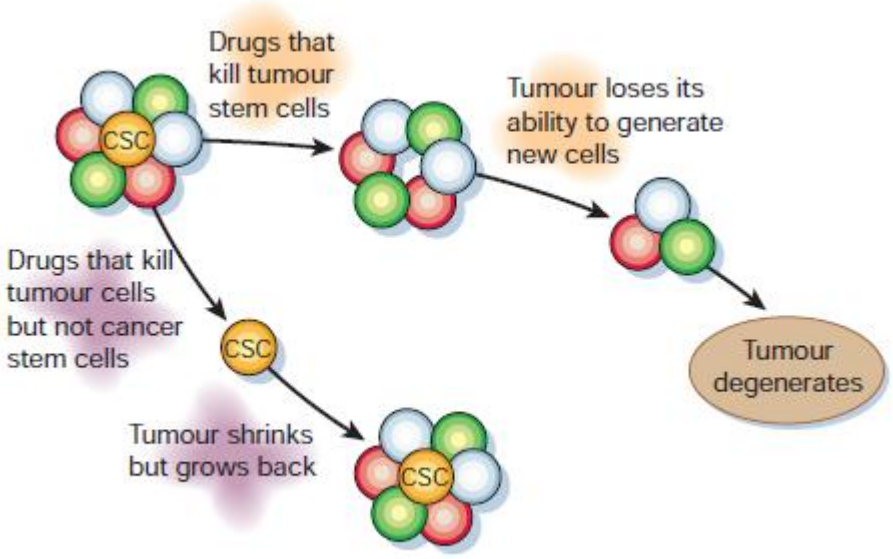

Figure 7: Conventional therapies may shrink tumours by killing mainly cells with limited proliferative potential. If the putative cancer stem cells are less sensitive to these therapies, then they will remain viable after therapy and re-establish the tumour. In the other way, if therapies can be targeted against cancer stem cells, then they might be more effectively killing the cancer stem cells, rendering the tumours unable to maintain themselves or grow. Thus, even if cancer stem cell-directed therapies do not shrink tumours initially, they may eventually lead to cures (Adapted from Reya et al., 2001).

The presence of active transmembrane ABC transporter family members, such as multidrug resistance transporter 1 (MDR1 - Multidrug Resistance Transporter 1) and ABCG2, can facilitate the efflux of drugs (Klonisch et al., 2008). In hematological malignancies, leukemic stem cell (LSC) phenotype-expressing subpopulations were found to overexpress several ABC drug efflux transporters that are implicated in drug resistance mechanisms and the LSC exhibited significantly higher efflux of some drugs

than tumour bulk components. (de Grouw et al., 2006; Wulf et al., 2001). The ABC transporters are the largest family of transmembrane proteins, and are responsible for the ATP-dependent movement of a wide variety of xenobiotics (including drugs), lipids and metabolic products across the plasma and intracellular membranes (Schatton et al., 2009). The ABC-transporter-encoding genes most extensively studied in stem cells are ABCB1 and ABCG2, and along with ABCC1, they represent the three major multidrug-resistance genes that have been identified in tumour cells (Dean et al., 2005).

Thus, as mentioned above, if CSCs are the only cells in a malignancy with the ability to expand and promote tumour growth, and, more importantly, with the capacity to metastasize, then it is urgent that novel cancer therapies target the CSCs population. Despite the great advantage that must outcome using chemotherapy against the CSCs, there are some key aspects that have to be considered. Such as, the identification of drugs that specifically target CSCs and not normal stem cells; assays should be developed that prevent, the very likely scenario, that those drugs do not target the normal tissue stem cells as well (Dalerba et al., 2007c; Diamandis et al., 2007). For this, it is essential to find ways to discriminate between normal- and CSCs. Moreover, the heterogeneity of the CSCs can be responsible for different patterns of resistances to therapy, which can result in the need to use more than one chemotherapy drug to efficiently eliminate them (Schatton et al., 2009).

Multiple lines of investigation indicate that several approaches might prove useful in enhancing clinical responses to systemic therapy through CSCs targeting, including CSCs ablation through monoclonal antibodies, blockade of CSCs functions, reversal of CSCs-associated resistance mechanisms, or induction of CSCs differentiation through epigenetic differentiation therapy. Therefore, combination therapies targeting CSCs and tumour bulk populations are most likely to lead to

optimized cancer treatments and to further reduce cancer morbidity and mortality in human patients (Boman et al., 2008; Schatton et al., 2009).

As stated above, there are several properties of the CSCs that can be responsible for their resistance to chemotherapy. Among them, there are alterations in the apoptotic pathways in the CSCs. Apoptosis is the ubiquitous and highly regulated mechanism by which cells undergo programmed death and a central regulator of normal tissue homeostasis (Degterev et al., 2003; Hamacher et al., 2008). The apoptotic process has an important role in many different stages of development and normal physiology, continuing to have an important role in postnatal organs (Thompson, 1995).

Acquisition of mechanisms to evade apoptosis is a hallmark of cancer, with both the loss-of-function of pro-apoptotic signals and gain-of-function of anti-apoptotic mechanisms contributing to tumourigenesis and the cancer phenotype (Hanahan and Weinberg, 2000). Defective apoptotic mechanisms allow genetically unstable cancer cells to avoid elimination and confer resistance to chemotherapy (Cummings et al., 2004; Schmitt, 2003; Schulze-Bergkamen and Krammer, 2004). As such, modulating the apoptotic pathways likely represents a propitious strategy for inducing tumour-cell death and increasing responses to chemotherapy, radiotherapy and even targeted therapies.

The execution of apoptosis relies on a group of cysteine proteases, the caspases (Degterev et al., 2003; Hager and Hanahan, 1999). There are two alternative pathways to initiate apoptosis and both finally activate the executioner caspases-3, -6 and -7 (Figure 8) (Hamacher et al., 2008). The first pathway is known as the intrinsic or mitochondrial pathway, because the mitochondria have a central role in the initiating of apoptosis. The exact mechanism of initiation by different apoptotic stimuli is still not entirely clear, but involves an imbalance of pro- apoptotic (e.g. Bax and Bak) and anti-

apoptotic (e.g. Bcl-2 and Bcl-XL) members of the BCL-2 protein family (Adams and Cory, 2007; Ziegler and Kung, 2008). This imbalance finally leads to the impairment of the integrity of the outer mitochondrial membrane (Hamacher et al., 2008). These apoptotic factors will activate the caspase-3, which cleaves poly-(ADP-ribose) polymerase (PARP) (Endres et al., 1997; Namura et al., 1998). PARP inactivation after cleavage by caspase-3 leads to DNA injury and subsequently to apoptotic cell death (Broughton et al., 2009). The second pathway is called the extrinsic pathway and is mediated by different death receptors on the cell surface (Debatin KM, Krammer, 2004; Fas et al., 2006; Wajant, 2007). Activation of the receptors after extracellular binding of the specific ligands (TNF-α, FAS-L and TRAIL) initiates the recruitment of FADD (FAS-associated death domain protein), procaspase-8 and -10 to the death domain, which are forming the DISC (death inducing signaling complex). At the DISC, the initiator caspase-8 is activated, which induce the executioner caspases (Hamacher et al., 2008).

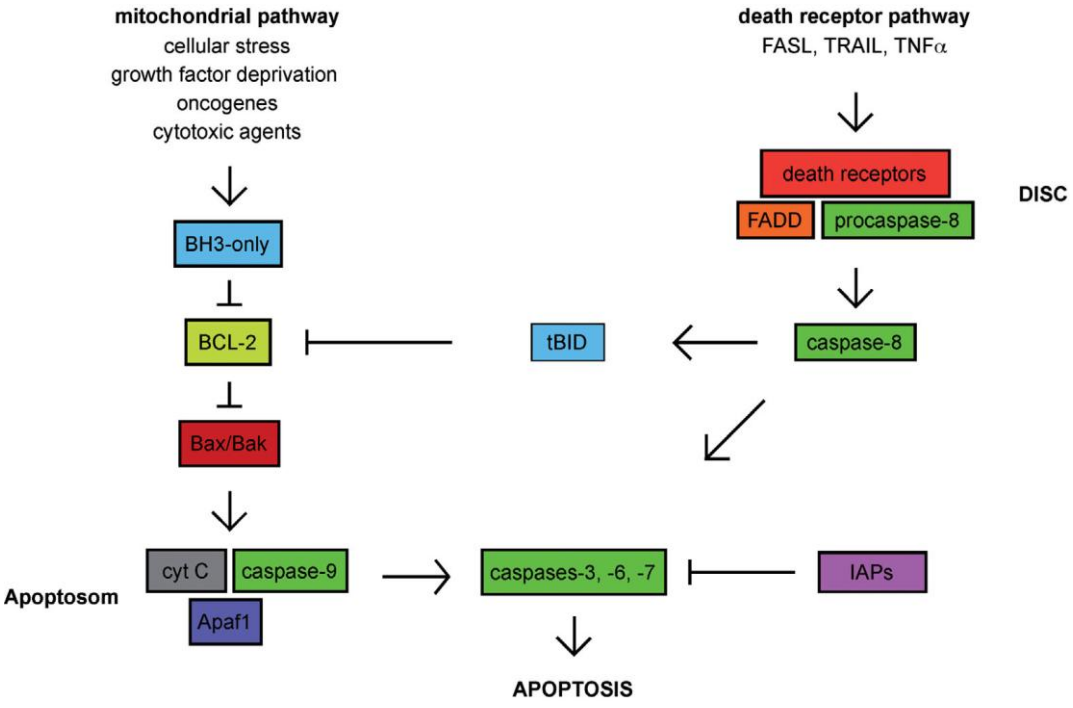

Figure 8: Pathways to Apoptosis. The mitochondrial pathway is activated by BH3-only proteins, which sense cellular stress and inactivate pro-survival

Bcl-2 family members. This leads to the permeabilization of the outer mitochondrial membrane and the release of cytochrome C, Apaf-1 and caspase-9. The death receptor pathway is activated by the TNF family ligands. Caspase-8 is activated by adaptor proteins including FADD. In PDAC cells, the death receptor pathway is linked to the mitochondria by the BH3-only protein Bid that is cleaved by caspase-8 (Adapted from Hamacher et al., 2008).

1.6 Objectives

The main objective proposed for this work is to clarify the involviment of gastric cancer stem cells (CSCs) in gastric cancer resistance to currently available drugs. Also, the identification of putative molecular mechanisms involved in chemoresistance of gastric CSCs was considered.

In order to accomplish this goal, the expression of CD44 (a putative marker of gastric CSCs) in AGS and MKN45 gastric cancer cell lines, is analyzed by flow cytometry, evaluating the existence of subpopulations of potential gastric CSCs. Furthermore, the subpopulation of gastric CSCs is separated by magnetic cell sorting (MACS) and the resistance of these gastric cancer cell lines and the AGS $CD44^+$ and $CD44^-$ subpopulations to the chemotherapy drugs (cisplatin and 5-FU) is evaluated.

Additionally, the expression patterns of some proteins involved in the apoptotic pathways are analyzed in the AGS and MKN45 cell lines as well as in the AGS $CD44^+$ and $CD44^-$ subpopulations.

2. Materials and Methods

2.1. Gastric adenocarcinoma AGS and MKN45 cell lines maintenance and culture conditions

During this work, AGS (CRL-1739, ATCC, Manassas, Virginia, USA) and MKN45 cell lines (obtained from Japanese Cancer Research Resources Bank, Tokyo, Japan), isolated from gastric adenocarcinomas were used as established models. These human cancer cell lines have similar characteristics, such as poorly differentiation, an epithelial morphology and are both adherents.

These cell lines were maintained in culture in RPMI (Roswell Park Memorial Institute medium) 1640 medium with 25 mM Hepes and GlutaMAX-1 (GIBCO™ Invitrogen Life Technologies) supplemented with 10% (v/v) fetal bovine serum inactivated (FBS) (GIBCO™ Invitrogen Life Technologies) and 0.1% of a gentamicine 50 mg/mL stock solution (GIBCO™ Invitrogen Life Technologies). Cells were maintained at 37 ºC, in a humidified atmosphere containing 5% CO_2. Whenever the cultures became confluent (80% of confluence) the cells were trypsinized with 0.05% trypsin/0.02% EDTA solution (GIBCO™ Invitrogen Life Technologies) and subcultured to fresh medium.

2.2. Analysis of the expression at the cell-surface of the CD44 by flow cytometry

The presence of CD44, a cell-surface glycoprotein, was assessed in both AGS and MKN45 cell lines by flow cytometry with an anti-CD44 – FITC antibody ((G44-26) monoclonal antibody (mouse) – BD Biosciences).

To perform the flow cytometry analysis, both MKN45 and AGS cell lines and AGS subpopulations $CD44^-$ and $CD44^+$ were trypsinized (5 min at 37ºC, using Tryple Express (GIBCO™ Invitrogen Life Technologies)) when 80% of confluence was reached. For each analysis, $1x10^6$ cells were used. The cells were centrifuged at 201x*g* for 5 minutes. The supernatant was discarded and the cells were washed with 2 mL of MACS buffer (PBS 1x with 2mM of EDTA and 1% of BSA). The pelleted cells were resuspended with an anti-CD44 - FITC antibody solution in a 1:5 dilution (100 µL of the MACS buffer and 20 µL of the antibody) for 20 minutes at 4˚C in the dark. After the incubation period, the cells were washed two times with MACS buffer. The supernatant was discarded and the pelleted cells were resuspended in 500 µL of MACS buffer, and analysed using the Calibur BD-Bioscience Flow Cytometer.

A negative control was carried out, in which the cells were subjected to the same procedure with the exception of antibody incubation.

The analysis of the results was performed using the software FlowJo version 7.2.5.

2.3. Separation of $CD44^-$ and $CD44^+$ subpopulations by Magnetic Cell Sorting (MACS)

In order to separate both AGS $CD44^+$ and AGS $CD44^-$ subpopulations the chosen method was MACS (Figure 9). The magnetic separation was performed using the MidiMACS™ magnetic separation kit of Miltenyi Biotec (Madrid, Spain) according to the recommendations of the manufacturer.

The cells were cultured to 80% of confluency in T75 cm^2 flaks, trypsinized (5 min at 37^oC, using Tryple Express) and counted using a Neubauer chamber. For the magnetic cell sorting, $1x10^7$ cells were used.

2.3.1. MACS – Direct magnetic separation with anti-CD44 microbeads

Cells were centrifuged at 201x*g* for 5 minutes, and then washed twice with MACS buffer. After another centrifugation, the pellet was resuspended in 1 mL of MACS buffer and the cell suspension was transferred to a previously hydrated pre-separation column (30 μm) (Milteny Biotec). The resulting suspension was then centrifuged at 201x*g* for 5 minutes and the supernatant was discarded. The cells were incubated for 20 minutes at 4˚C in the dark with 20 μL of anti-CD44 microbeads (Milteny Biotec) after being resuspended in 60 μL of MACS buffer supplied with 20 μL of FcR blocking reagent (Milteny Biotec). After incubation, 1 mL of MACS buffer was added and this suspension was centrifuged at 229x*g* for 10 minutes at 4˚C. The washed cells were resuspended in 500 μL of MACS buffer. The cell suspension was transferred to a LS column (previously hydrated with 3 mL of MACS buffer) (Milteny Biotec) placed in a magnetic support. The total effluent was collected as the $CD44^-$ fraction and the column was washed three times with 3 mL of MACS buffer.

The column was then removed from the magnet and, with the aid of a plunger, 5 mL of MACS buffer were used to flush the microbead labelled cells out of the column. The effluent was collected as the $CD44^+$ fraction.

The $CD44^-$ subpopulation was subsequently passed through the LS column and washed three times with 1 mL of MACS buffer further to deplete remaining $CD44^+$ cells. Both $CD44^+$ and $CD44^-$ fractions were centrifuged at 201x*g* for 5 minutes and the

pellet was resuspended in 3 mL of RPMI 1640 medium with 25 mM Hepes and GlutaMAX-1 supplied with 10% (v/v) FBS and 0.1% of a gentamicine 50 mg/mL stock solution and transferred to a new T75 cm^2 flask containing fresh culture medium. The AGS $CD44^+$ and $CD44^-$ subpopulations were then maintained at 37 ºC, in a humidified atmosphere containing 5% CO_2.

2.3.2. MACS – Indirect magnetic separation with anti-FITC microbeads

Indirect magnetic separation of the AGS $CD44^+$ and $CD44^-$ subpopulations was done, recurring to anti-FITC microbeads (Milteny Biotec), which have the ability to specifically bind to the anti-CD44 - FITC antibody. $1x10^7$ cells were centrifuged at 201x*g* for 5 minutes and then washed twice with MACS buffer, two times. After another centrifugation, the pellet was resuspended in 1 mL of MACS buffer and the cell suspension was passed through a pre-separation column (30 μm).

The resulting suspension was then centrifuged at 201x*g* for 5 minutes and the supernatant was discarded. The cells were resuspended in a 1:10 diluted anti-CD44 - FITC antibody solution in MACS buffer. The suspension was incubated for 20 minutes at 4˚C in the dark. After incubation, 1 mL of MACS buffer was added and the suspension was centrifuged at 229x*g* for 10 minutes at 4˚C. The washed cells were resuspended in 80 μL of MACS buffer supplied with 10 μL of FcR blocking reagent and 10 μL of the anti-FITC microbeads. The suspension was incubated for 15 minutes at 4˚C in the dark. After the incubation period, the cell suspension was washed with 1 mL of MACS buffer and centrifuged at 229x*g* for 10 minutes and the cells resuspended in 500 μL of MACS buffer. The cell suspension was transferred to a LS column (previously hydrated with 3 mL of MACS buffer) placed in a magnetic support. The

total effluent was collected as the $CD44^-$ fraction and the column was washed three times with 3 mL of MACS buffer. The LS column was then removed from the magnet and, with the aid of a plunger, 5 mL of MACS buffer were used to flush the microbead labelled cells out of the column. The effluent was collected as the $CD44^+$ fraction.

The $CD44^-$ subpopulation was subsequently passed through the LS column and washed three times with 1 mL of MACS buffer further to deplete remaining $CD44^+$ cells. Both $CD44^+$ and $CD44^-$ fractions were centrifuged at 201x*g* for 5 minutes and the pellet was resuspended in 3 mL of RPMI 1640 medium with 25 mM Hepes and GlutaMAX-1 supplied with 10% (v/v) fetal bovine serum inactivated and 0.1% of a gentamicine 50 mg/mL stock solution and transferred to a new T75 flask containing fresh culture medium. The AGS $CD44^+$ and $CD44^-$ subpopulations were then maintained at 37 ºC, in a humidified atmosphere containing 5% CO_2.

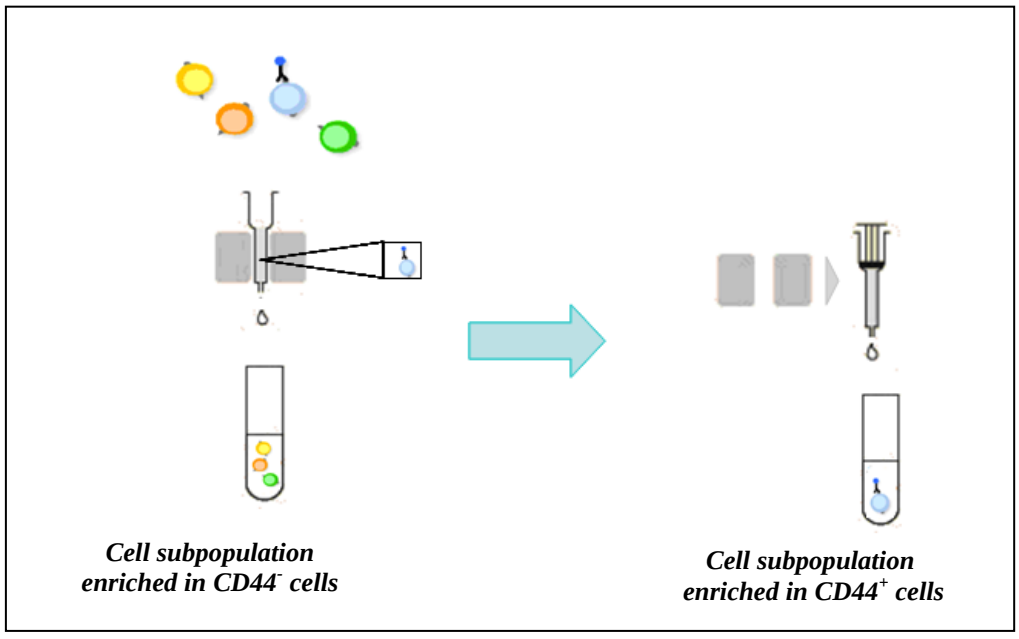

Figure 9. Schematic representation of the method of magnetic activated cell sorting (MACS).

2.4. Evaluation of the cytotoxic effects of 5-FU and cisplatin in AGS and MKN45 cells by the Sulforhodamine B colorimetric assay

The Sulforhodamine B colorimetric assay (SRB) (in vitro Toxicology Assay Kit – Sigma-Aldrich) was used to determine the GI50 values of different drugs, hence their cytotoxic efficiency on the studied cell lines. This assay, mostly used for cytotoxicity screening, was developed in 1990 and relies on the ability of SRB to bind protein components of cells that have been fixed with trichloroacetic acid (TCA). Sulforhodamine B is a bright pink aminoaxanthe dye which contains two sulfonic groups that bind to basic amino-acid residues under basic conditions. As the binding of SRB is stoichiometric, the amount of dye extracted from stained cells is directly proportional to the cell mass (Vichai and Kirtikara, 2006).

2.4.1. GI50 values determination

In order to evaluat the effect of the chemotherapy drugs in cell growth, the MKN45 and AGS cell lines, as well as AGS subpopulations $CD44^-$ and $CD44^+$ were seeded in triplicate for each drug treatment on 96-well plates. Regarding the AGS cell line and the respective $CD44^-$ and $CD44^+$ subpopulations, $2x10^3$ cells/well were plated in 100 µL of culture medium (RPMI 1640 medium with 25 mM Hepes and GlutaMAX-1*)* supplemented with 10% (v/v) FBS. The same was done with MKN45 cells but, in this case, they were plated in 200 µL of culture medium.

The 96-well plates were incubated in a humidified atmosphere containing 5% CO_2, at 37 ºC for 24 hours.

After this period, the cells were treated with two different drugs, 5-Fluorouracil (5-FU) (Sigma-Aldrich, Sintra, Portugal) and cisplatin (Sigma-Aldrich). These pharmacological compounds were administrated to the cells in a range of concentrations from 0.01 to 100 µM. To the AGS cell line and their subpopulations ($CD44^-$ and $CD44^+$) 100 µL of the tested drug (5-FU or cisplatin) solution (diluted with fresh culture medium in order to obtain the desired concentration) were added to each well, whereas for MKN45 200 µL of drug solution were applied. Controls were conducted in all trials, consisting in wells containing cells not subjected to any drug treatment, and wells containing just culture medium.

All the cells lines were incubated with the desired drug for 48h in a humidified atmosphere containing 5% CO_2, at 37 ºC.

After the drug treatment, the cells were fixed *in situ* by adding 50 µL of cold 50% (w/v) TCA (final concentration, 10% TCA) to the cell line MKN45 and 25 µL of cold 50% TCA for the AGS cells and their subpopulations, followed by an incubation period of 60 minutes at 4 ºC. The supernatant was then discarded, and the plates were washed three times with water and dried at room temperature. After the fixation procedure, 50 µL of SRB solution was added to each well, and the plates were incubated for 30 minutes at room temperature. The unbound SRB was then removed by washing three times with 1% acid acetic (100 µL of acid acetic were added to each well) and the plates were air-dried.

Finally, the bound stain was solubilised with Tris buffer at 10 mM (pH 10.5) by adding 100 µL of Tris buffer to each well and shaking for 5 minutes at room temperature. The optical densities (OD) were determined on a spectrophotometric plate reader (Bio-Rad, model 680 microplate reader) at two wavelengths, 560 and 655 nm. The obtained data were analyzed and interpreted and the GI50 (50% growth inhibition

concentration) of each pharmacological compound for each one of the wild-type AGS and MKN45 cell lines and AGS $CD44^-$ and $CD44^+$ subpopulations was determined.

The GI50 values were calculated according to the equation GI50 = 100 x [(T - T0)/(C - T0)], in which T is the OD after exposure to a certain concentration of a drug, T0 is the OD at the start of drug exposure and C is the OD of the control (Yamori et al., 1999).

2.5. Statistical analysis

The presented graphs and statistical analysis were performed in Graph Pad Prism 5 software. Statistical significance was assessed by Student's *t*-test analysis. Results are presented as normalized means ± SD.

2.6. Immunoblotting

With the purpose of assessing and semi-quantify the expression of several proteins involved in the apoptosis pathways, namely Bcl-xl, Bcl-2, PARP and Caspase-3, the Western Blotting technique was performed. As a reference control, β-actin, a constitutively expressed protein in human cells - encoded by a housekeeping gene – was chosen, thus all membranes were incubated with anti-β-actin antibody.

2.6.1. Total protein extraction from the cell cultures

The protein extraction was performed when cells presented 80% of confluence. The extraction from the AGS cell line was performed at the same time as the

subpopulations $CD44^+$ and $CD44^-$ that were obtained by MACS. For these subpopulations the protein extractions were performed at the first passage.

To prepare the whole-cell extract, the cells were washed twice with cold phosphate buffer saline (PBS – 137 mM NaCl, 2.7 mM KCl, 1.8 mM K2PO4, 10 mM Na2HPO4·H2O, pH 7.4) and suspended in a CelLytic^{TM}M Cell Lysis Reagent (Sigma-Aldrich) containing protease inhibitors (Complete Protease Inhibitor Cocktail Tablets – Roche Applied Science, Carnaxide, Portugal) and vigorously scraped on ice. The cell suspension was incubated for 20 minutes at 4 ˚C under agitation, to lyse the cells. Then, the lysates were cleared of their insoluble fraction by centrifugation (100x*g*, 30 min, 4 ºC).

The protein content was determined using the bicinchoninic acid (BCA) method (BCA – 250 Protein Assay Kit – Bio-Rad, Amadora, Portugal), according to the recommendations of the manufacturer.

2.6.2. SDS-PAGE (Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis)

SDS-PAGE of whole-cell proteins was performed as described by Laemmli (1970). Proteins were separated by polyacrylamide gel electrophoresis (10% (v/v) bis-acrylamide) (Bio-Rad) in running buffer (25 mM Tris Base, 100 mM glycine, 0.1% (w/v) SDS, pH 8.8) during 2 hours at a current of 100 V. Protein denaturation was managed by intense heating for 10 minutes at 100 ˚C. Sample buffer (DTT + NuPAGE 4x (Invitrogen) (1:25)) in a proportion of 1:4 was added to 25 µg of deanatured protein extract and the obtained final volume of 24 µL was loaded into each well. Another well

was loaded with 10 µL of a protein molecular weight marker (Precision Plus Dual Color – Bio-Rad).

2.6.3. Electrotransference to the membrane

After the SDS-PAGE, the proteins were bloted to a PVDF (polyvinylidene difluoride) membrane (GE Healthcare, Carnaxide, Portugal), previously activated in methanol for 30 seconds, at 100 V for 1h and 30 minutes. The transfer buffer consisted in 0.25 M Tris base, 1.91 M Glycine, pH 8.8, supplemented with 20% (v/v) methanol, and the blotting was done in a MiniPROTEAN® 3 system (Bio-Rad, Amadora, Portugal).

2.6.4. Immunodetection

After the blotting, the membrane was incubated with the blocking solution PBS-Tween (PBS 1x + 0.01% Tween 20 (Sigma-Aldrich)) with 5% (w/v) low-fat dry milk for 1 hour at room temperature. Then, the membrane was cut, taking into account the molecular weight of the proteins in study.

The primary antibodies used for immunoblotting were: anti-Bcl-xl (H-62) (Santa Cruz Biotechnology, Santa Cruz, USA), anti-Bcl-2 (124) (Dako, Denmark), anti-PARP (H-250) (Santa Cruz Biotechnology) and anti-Caspase-3 (4-1-18) (Upstate biotechnology, New York, USA) (table III). The different membranes were incubated with the corresponding primary antibody diluted in PBS-Tween supplemented with 5% (w/v) low-fat dry milk, overnight at 4 °C, with gentle shaking. The membranes were then washed three times for 10 minutes with PBS-Tween at room temperature and

subsequently incubated with suitable Horseradish Peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology), either goat anti-mouse or goat anti-rabbit depending on the primary antibody, diluted 1:2000 in PBS-Tween with 5% (w/v) low-fat dry milk. This incubation was performed for 1 hour at room temperature. Membranes were again washed with PBS-Tween (3 times, 10 minutes each) and the signals were detected using ECL (Imuno-Star HRP Peroxidase Buffer –Bio-Rad), according to the manufacturer's instructions. Finally, the membranes were revealed in photographic films (Kodak – BioMax Film) with varied time of exposure depending on the primary antibody used.

Table III: Primary and secondary antibodies used and their dilutions.

Primary Antibody	***Dilution***	***Secondary Antibody (1:2000)***
Anti-Bcl-xL	1:200	Goat anti-rabbit
Anti-Bcl-2	1:100	Goat anti-mouse
Anti-Caspase 3	1:2000	Goat anti-mouse
Anti-PARP	1:4000	Goat anti-rabbit
Anti-β-actina	1:2000	Goat anti-mouse

2.6.5. Stripping and reprobing

In order to reprobe the membranes for β-actin immunodetection, they were stripped with stripping buffer (1 M Glycine and 20% SDS) for 30 minutes and then the membranes were washed two times with PBS-Tween for 10 minutes.

Membranes were blocked for 1 h in PBS-Tween with 5% low-fat dry milk and then reprobed with the primary antibody, anti-β-actin (A-15) (Santa Cruz Biotechnology) with gentle shaking for 1 hour at room temperature. After incubation, the membranes were washed with the same solution used above (3 times, 10 minutes each), and then were incubated with secondary antibody (Horseradish Peroxidase-conjugated goat anti-mouse). Membranes were again washed with PBS-Tween (3 times, 10 minutes each) and the signals were detected using ECL (Imuno-Star HRP Peroxidase Buffer), according to the manufacturer's instructions. Finally, the membranes were revealed in photographic films with varied time of exposure depending on the primary antibody used.

3. Results

In order to evaluate the potential existence of cancer stem cells (CSCs) in gastric cancer cell lines, as well as their influence on the resistance to chemotherapy two gastric cancer cell lines at the same stage of differentiation were analyzed: AGS and MKN45 cells. The resistance of these cell lines to cisplatin and 5-flourouracil (5-FU), drugs commonly used in chemotherapy of gastric cancer was assessed by the determination of the drug concentration inhibiting 50% of the growth of the cancer cells, designated as the GI50 value.

Furthermore, the expression at the cell-surface of the CD44 glycoprotein was analyzed by flow cytometry, in the AGS and MKN45 cell lines, with the aim of isolating the potential CSCs subpopulations, previously described as expressing significantly higher amounts of CD44. In order to separate the $CD44^-$ and $CD44^+$ subpopulations from the WT AGS cell population, we used the magnetic cell sorting (MACS) technique. Two methods of magnetic separation were performed: a direct magnetic separation, using anti-CD44 microbeads, and an indirect separation, with anti-FITC microbeads, with the cells previously labelled with the anti-CD44 - FITC antibody. This type of separation, recurring to the same antibody that was used to analyze the expression of CD44 in the gastric cancer cell lines, has the advantage, comparatively with the direct magnetic separation, of allowing to know that about 30% of the AGS cell population will be isolated as the potential $CD44^+$ subpopulation, since this was the percentage of cells expressing the CD44 glycoprotein. After the separation, the GI50 values of cisplatin and 5-FU for the respective $CD44^-$ and $CD44^+$ subpopulations were also determined.

Due to time constrains we also performed a parallel study, aiming to characterize by immunoblotting, the expression levels of some proteins involved in the apoptotic pathways in the MKN45 and AGS WT cells, and both AGS $CD44^-$ and $CD44^+$

subpopulations. Pro- and anti-apoptotic proteins were chosen in order to evaluate the possible contribution of an apoptosis inhibition towards the chemoresistant phenotype of the $CD44^+$ subpopulation, and to infer about possible differences in relation to WT and $CD44^-$ AGS cells. The apoptotic proteins studied were Bcl-2, Bcl-XL, PARP and caspase-3.

The Bcl-2 family of proteins plays a central role in the regulation of apoptosis in a cell. Members of this family, the Bcl-XL and the Bcl-2 protein are key anti-apoptotic proteins (Minn et al., 1999; Yang et al., 1997). Another protein identified as being a key mediator of apoptosis is capase-3, an effector caspase that is activated by initiator caspases (Ziegler and Kung, 2008). Together with these proteins, the Poly (ADP-ribose) polymerase (PARP) protein has also been studied. Full-length PARP is a 116kDa protein involved in the repair of DNA, in differentiation and in chromatin structure formation. During apoptosis this protein is cleaved by caspase-3, and possibly by other caspases, into an 89kDa fragment (Boulares et al., 1999). The cleavage of PARP inactivates it and inhibits PARP's DNA-repairing abilities, constituting an apoptotic marker.

3.1. Determination of the GI50 values of cisplatin and 5-FU for the AGS and MKN45 cell lines

With the purpose of evaluating the citotoxicity of cisplatin and 5-FU in the cell growth, the GI50 values were determined using the sulforhodamine B colorimetric assay. In these assays, the MKN45 cells were exposed to 5-FU or cisplatin in a concentration range of 0.01 to 100 µM for both drugs. On the other hand, the AGS cells

were subjected to 5-FU or cisplatin in the concentration ranges of 0 to 15 µM and 0 to 25 µM (Figure 10 and 11).

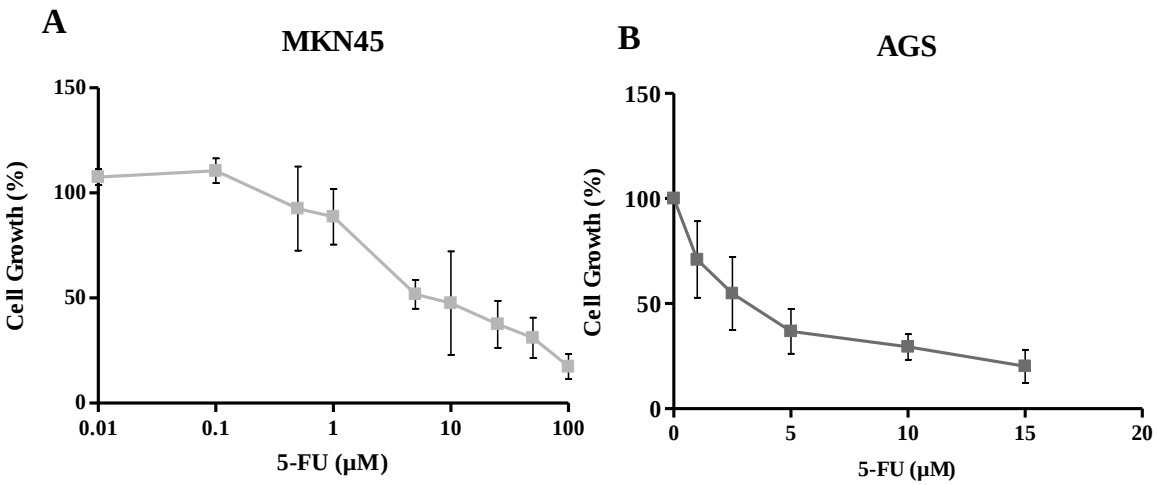

Figure 10: Graphic representation of the dose-response cytotoxicity (effect in cell growth inhibition) of 5-FU after 48h of treatment in MKN45 (**A**) and AGS (**B**) gastric cancer cell lines. The GI50 values were determined by SRB assay. The results are presented as the mean of at least three independent experiences (± standard deviation). The X axis of the data **A** is presented in logarithm scale.

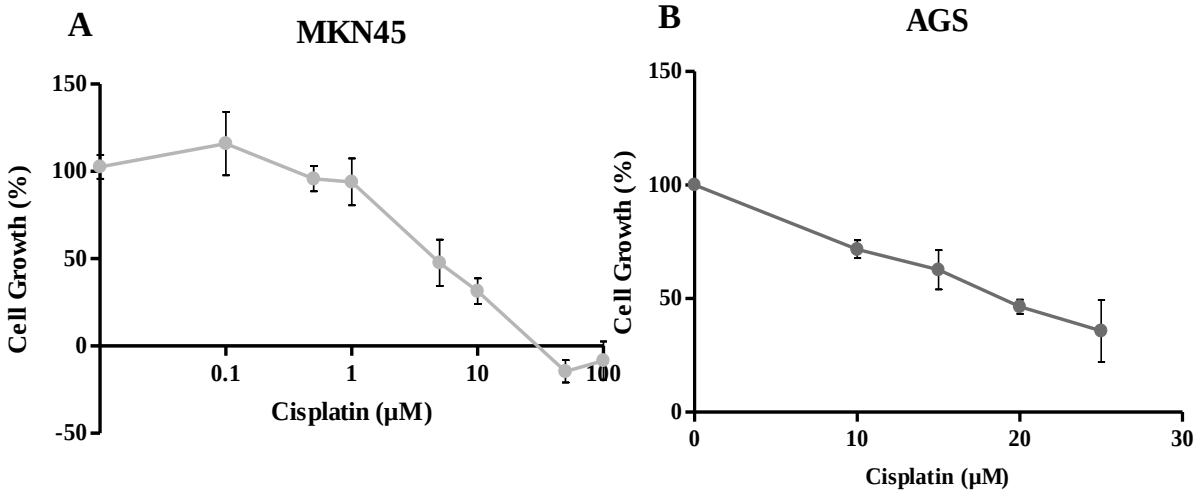

Figure 11: Graphic representation of the dose-response cytotoxicity of cisplatin after 48h of treatment in MKN45 (**A**) and AGS (**B**) gastric cancer

cell lines. The GI50 values were determined by SRB assay. The results are presented as the mean of at least three independent experiences (± standard deviation). The X axis in the data **A** is presented in logarithm scale.

When treated with cisplatin, after a 48h exposure period, the MKN45 cell line displayed 50% of inhibition in its cell growth when the drug concentration reached 4.69 ± 1.94 µM. For the same cell line, the calculated GI50 value was 5.42 ± 1.69 µM for 5-FU. The determined GI50 values for each drug, for both AGS and MKN45 cell lines are depicted in figure 12.

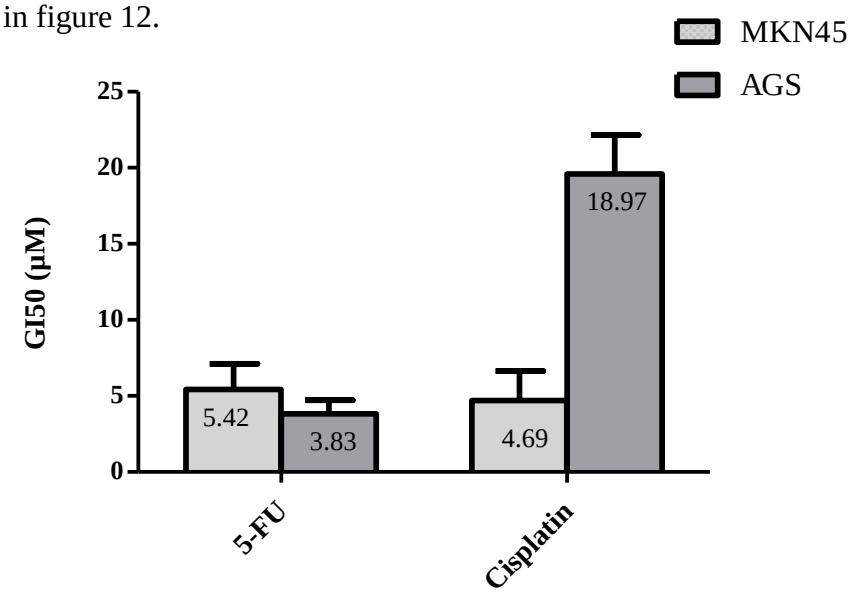

Figure 12: Graphic representation of the values of the drug concentration that inhibited cell proliferation by 50% (GI50) of MKN45 and AGS gastric cancer cell lines as a response to 5-FU and cisplatin. The GI50 values were determined by SRB assay. The results are presented as the mean of at least three independent experiences (± standard deviation).

On the other hand, as demonstrated in figure 13, AGS WT cells displayed a higher resistance to cisplatin than to 5-FU. In this cell line, the GI50 value of cisplatin was much higher than the one of MKN45 cells, presenting a value of 18.97 ± 2.48 µM,

while 5-FU showed a GI50 of 3.83 ± 0.88 µM, a value more similar to the one displayed by the MKN45 cell line.

3.2. Analysis of the expression of the CD44 protein by flow cytometry

In order to evaluate the potential existence of a subpopulation of CSCs from the MKN45 and AGS gastric cancer cell lines, the expression at the cell-surface of the CD44 protein was analyzed by flow cytometry. This glycoprotein was previously described as a marker for the subpopulation of gastric CSCs in several human gastric cancer cell lines (Takaishi et al., 2009).

The results demonstrated a variation in the levels of expression of the CD44 marker, in the two gastric cancer cell lines subject of our study. In the case of the MKN45 cell line a remarkable expression of CD44 of 99.15% was observed (Figure 13), whereas the AGS cell line showed only about 29.06% of expression of the marker at the cell-surface (Figure 14).

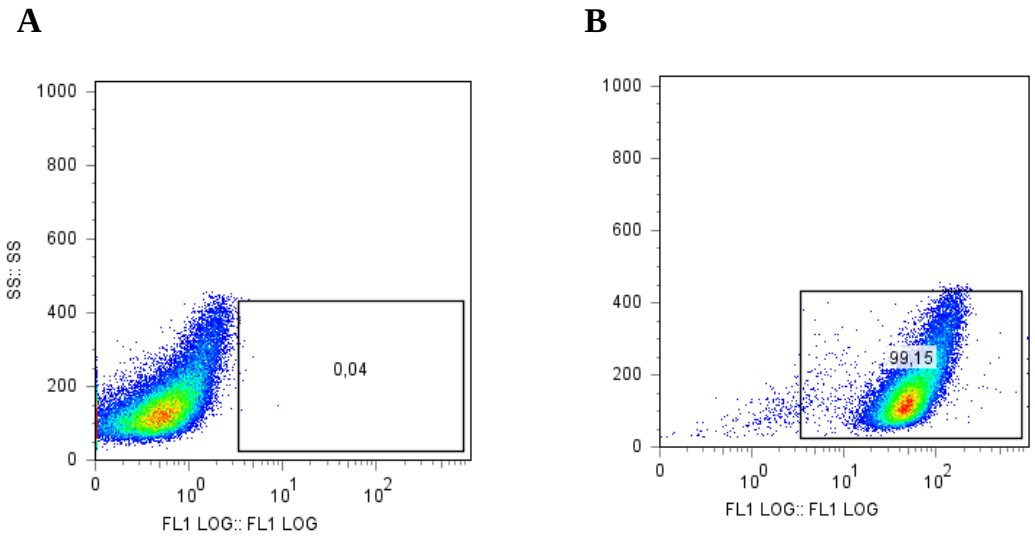

Figure 13: Analysis of the expression of the cell-surface protein CD44 in MKN45 gastric cancer cell line by flow cytometry. (**A**) Population of cells

unstained (control). (**B**) Population of cells stained with the anti-CD44 – FITC antibody.

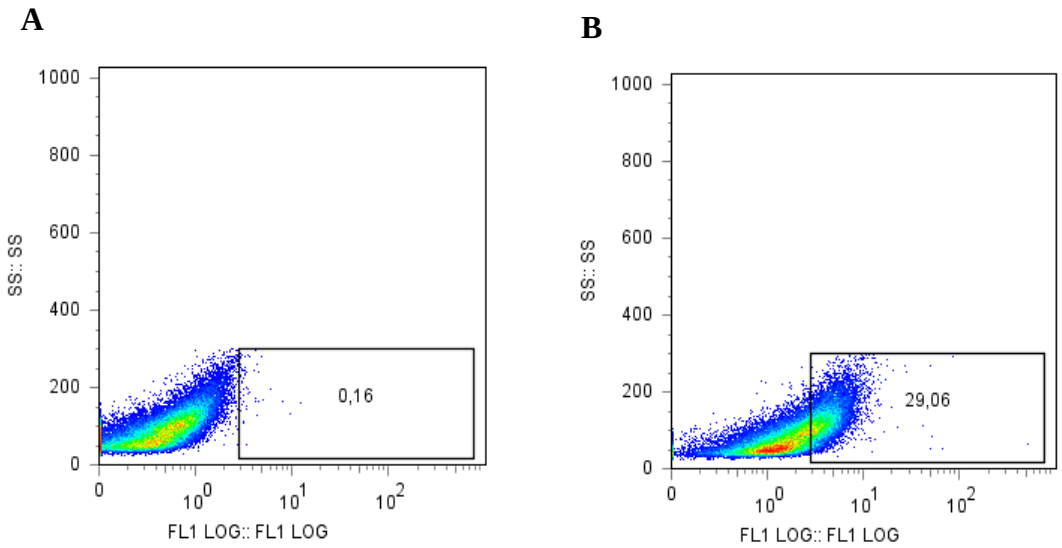

Figure 14: Analysis of the expression of the cell-surface protein CD44 in AGS gastric cancer cell line by flow cytometry. (**A**) Population of cells unstained (control). (**B**) Population of cells stained with the anti-CD44 – FITC antibody.

Such significant difference in the expression of CD44 protein at the cell-surface between both cell lines are undoubtedly interesting results, taking into account that MKN45 and AGS are both gastric cancer cell lines sharing some significant common characteristics.

3.3. Evaluation of the resistance to cisplatin and 5-FU of the AGS $CD44^+$ and $CD44^-$ subpopulations

The $CD44^+$ (enriched in CSCs) and $CD44^-$ subpopulations were obtained by magnetic cell sorting from the AGS gastric cancer cell line. The separation was performed in two ways, by direct and indirect magnetic separation (MACS), as mentioned above.

The $CD44^+$ and $CD44^-$ subpopulations obtained by the two methods of separation were subjected to the anticancer drugs (5-FU and cisplatin) for 48h, with the goal of assessing the resistance of the potential subpopulation of CSCs. To determine this resistance the GI50 values were calculated for each drug.

In the subpopulations separated with the anti-CD44 microbeads, the GI50 values of 5-FU were 3.83 ± 0.88 µM for the WT population, 4.89 ± 2.02 µM for the subpopulation $CD44^-$ and 4.75 ± 2.70 µM for the subpopulation enriched in CSCs (Figure 15 (**A**)). On the other hand, after cisplatin exposure, a GI50 value of 18.97 ± 2.48 µM was determined for the WT population, whereas for the subpopulations $CD44^-$ and $CD44^+$ GI50 values of 15.24 ± 1.05 µM and 15.46 ± 2.96 µM of cisplatin were, respectively (Figure 15 (**B**)). For 5-FU no significant statistical difference between any of the subpopulations was observed, after analysis by Student's t test (p-value > 0.05). In the case of cisplatin, no significant statistical difference between the $CD44^-$ and $CD44^+$ subpopulations and between the population WT and $CD44^+$ subpopulation was showed (p-value > 0.05). But, a significant statistical difference comparing the $CD44^-$ subpopulation and the population WT was observed, after analysis by Student's t test (p-value <0.05).

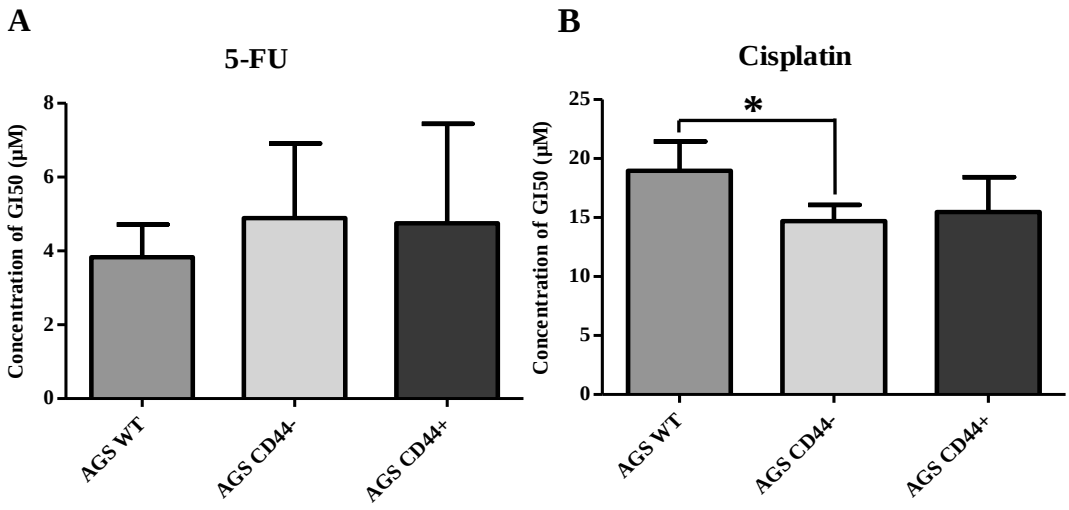

Figure 15: Representation of the GI50 values of 5-FU and cisplatin for the AGS cell line (WT population) and the AGS $CD44^-$ and AGS $CD44^+$ subpopulations separated with the anti-CD44 microbeads. (**A**) GI50 values of 5-FU for the WT population and the $CD44^-$ and $CD44^+$ subpopulations, after a 48h treatment. (**B**) GI50 values of cisplatin for the WT population and the $CD44^-$ and $CD44^+$ subpopulations, after a 48h treatment. Bars represent the mean ± SD of at least three independent experiments. Statistical analysis was performed with the Student's *t*-test (**p*-value = 0.0239).

In the subpopulations obtained by indirect separation, the GI50 values of 5-FU were 3.83 ± 0.88 µM for the WT population, 4.36 ± 0.36 µM for the subpopulation $CD44^-$ and 3.71 µM for the $CD44^+$ subpopulation as demonstrated in Figure 16 (**A**). The GI50 values in the cells treated with cisplatin were 18.97 ± 2.48 µM for the WT population, 14.67 ± 2.28 µM for the $CD44^-$ subpopulation and 14.31 ± 3.09 µM cisplatin for the subpopulation enriched in CSCs (Figure 16 (**B**)). For 5-FU no significant statistical difference between any of the subpopulations was observed, after analysis by Student's t test (*p*-value > 0.05). In the case of cisplatin, no significant statistical difference between the $CD44^-$ and $CD44^+$ subpopulations was observed, after

analysis by Student's t test (p-value > 0.05). The same was obtained when comparing results between the WT population and $CD44^+$ sub-population. However the p-value was suggestive (p-value = 0.0571). Finally, a significant statistical difference between the $CD44^-$ subpopulation and the population WT was observed, after analysis by Student's t test (p-value <0.05).

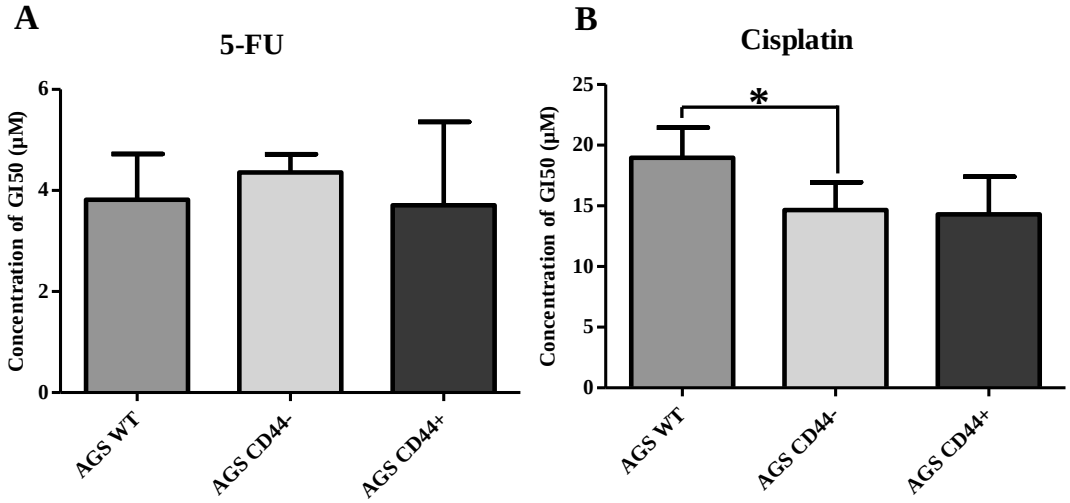

Figure 16: Representation of the GI50 values of 5-FU and cisplatin for the AGS cell line (WT population) and the AGS $CD44^-$ and AGS $CD44^+$ subpolations obtained by indirect separation with the anti-FITC microbeads. (**A**) GI50 values of 5-FU for the WT population and the $CD44^-$ and $CD44^+$ subpolations, after a 48h treatment. (**B**) GI50 values of cisplatin for the WT population and the $CD44^-$ and $CD44^+$ subpolations, after a 48h treatment. Bars represent the mean ± SD of at least three independent experiments. Statistical analysis was performed with the Student's *t*-test (**p*-value = 0.0432).

3.4. Analysis of the expression of apoptosis-related proteins in AGS WT cells, AGS CD44$^-$ and CD44$^+$ subpopulations and in MKN45 WT cells

In order to evaluate possible differences in the expression patterns of some proteins involved in apoptotic pathways (pro- and anti-apoptotic proteins) western blotting technique was performed. The expression of the Bcl-2, Bcl-XL, PARP and caspase-3 proteins, in AGS WT cells and their CD44$^-$ and CD44$^+$ subpolations was analyzed.

Immunoblotting of the Bcl-XL showed that the levels of this protein were virtually the same between the CD44$^-$ and CD44$^+$ subpolations and the WT cells for both methods of magnetic sorting (Figure 17).

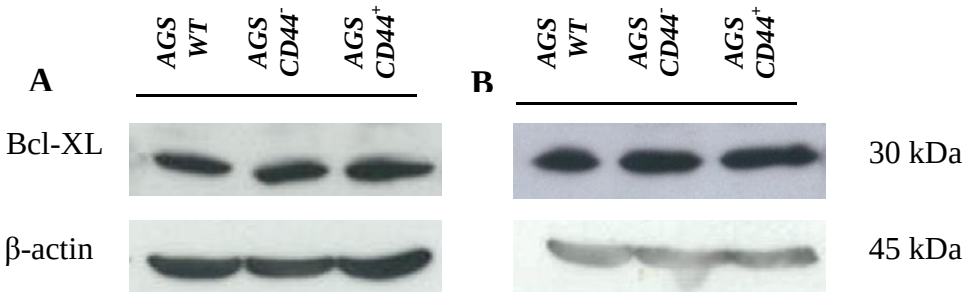

Figure 17: Western blot analysis of Bcl-XL protein expression in AGS WT cell line and in CD44$^-$ and CD44$^+$ subpolations. (**A**) The AGS CD44$^-$ and AGS CD44$^+$ subpolations were separated with the anti-CD44 microbeads. (**B**) The subpolations AGS CD44$^-$ and AGS CD44$^+$ obtained by indirect separation. Equal loading was confirmed with β-actin immunoblotting.

In the case of the Bcl-2 protein, the western blot demonstrated that the levels of this protein were very similar between the CD44$^-$ and CD44$^+$ subpolations and the WT

cells, whether the subpolations were isolated by indirect or direct magnetic separation (Figure 18).

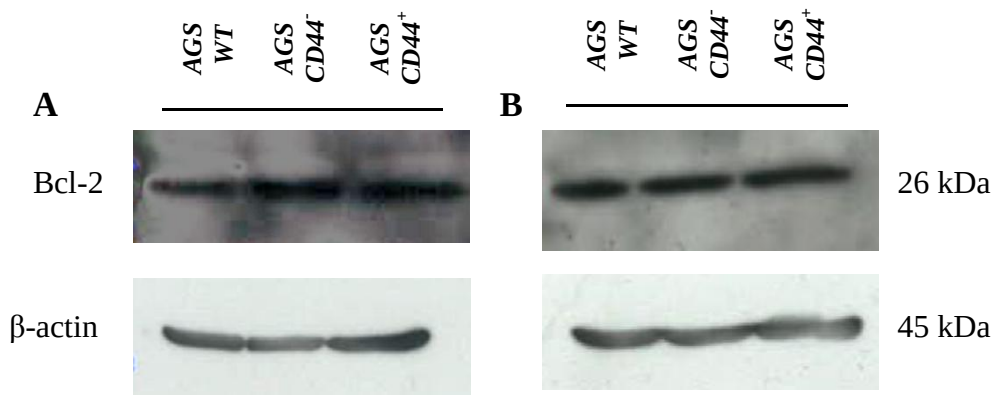

Figure 18: Western blot analysis of Bcl-2 protein expression in AGS WT cell line and in $CD44^-$ and $CD44^+$ subpolations. (**A**) The AGS $CD44^-$ and AGS $CD44^+$ subpolations were separated with the anti-CD44 microbeads. (**B**) The subpolations AGS $CD44^-$ and AGS $CD44^+$ obtained by indirect separation. Equal loading was confirmed with β-actin immunoblotting.

Caspase-3 protein levels were also assessed by western blot. In the both methods of separation, and comparatively between the AGS WT cells and $CD44^-$ and $CD44^+$ subpolations, no significant differences were observed in caspase-3 protein levels, as demonstrated in Figure 19.

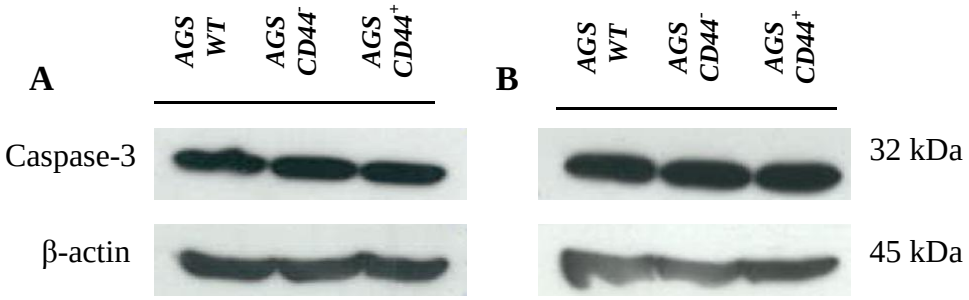

Figure 19: Western blot analysis of caspase-3 protein expression in AGS WT cell line and in $CD44^-$ and $CD44^+$ subpolations. (**A**) The AGS $CD44^-$ and AGS $CD44^+$ subpolations were separated with the anti-CD44 microbeads. (**B**) The subpolations AGS $CD44^-$ and AGS $CD44^+$ obtained by indirect separation. Equal loading was confirmed with β-actin immunoblotting.

Additionally, PARP protein levels were also evaluated. The antibody used in the blotting of this protein is unspecific to the cleaved and non-cleaved forms, detecting both forms. In the case of the $CD44^-$ and the $CD44^+$ subpolations obtained by direct separation, higher levels of PARP were observed in the AGS $CD44^+$ subpopulation (++) when compared with the $CD44^-$ subpolation (+) and the WT cells (-) (Figure 20 (**A**)). These results were observed in both forms, being that the cleaved form with 89 kDa presented a higher level of expression than the non-cleaved form. On the other hand, when isolated by indirect magnetic separation the protein amounts of both full-length and cleaved PARP, did not differ much between all the subpolations as shown in Figure 20 (**B**)).

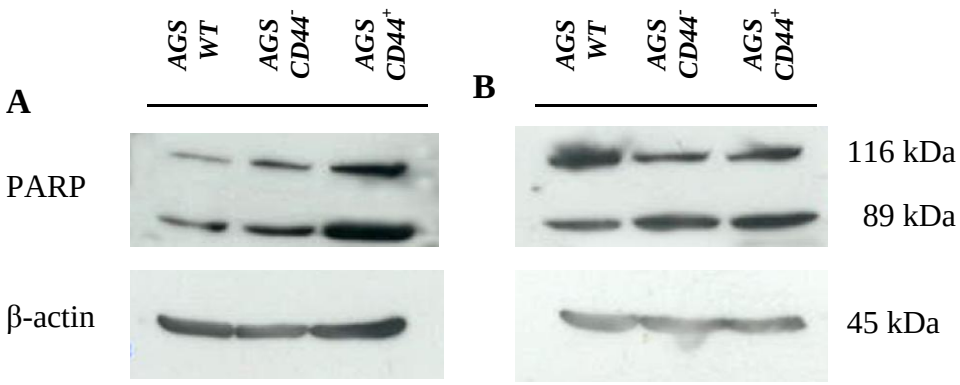

Figure 20: Western blot analysis of full-length and cleaved PARP protein expression in AGS WT cell line and in $CD44^-$ and $CD44^+$ subpolations. (**A**) The AGS $CD44^-$ and AGS $CD44^+$ subpolations were separated with the anti-CD44 microbeads. (**B**) The subpolations AGS $CD44^-$ and AGS $CD44^+$ obtained by indirect separation. Equal loading was confirmed with β-actin immunoblotting.

In addition, the expression levels of the same proteins analyzed for the AGS WT and their subpopulations ($CD44^-$ and $CD44^+$) were also assessed for the MKN45 WT cell line, allowing a comparison between the protein expression profiles of both cell lines. The obtained results, demonstrated in Figure 21 (**A**), showed a higher expression of Bcl-XL in the MKN45 cell line comparatively with the AGS WT cell line. On the other hand, the analysis of the expression of Bcl-2 demonstrated an absence of expression of this protein in the MKN45 cell line, contrarily to what was observed in the AGS WT cell line which expresses significant levels of Bcl-2 (Figure 21 (**B**)).

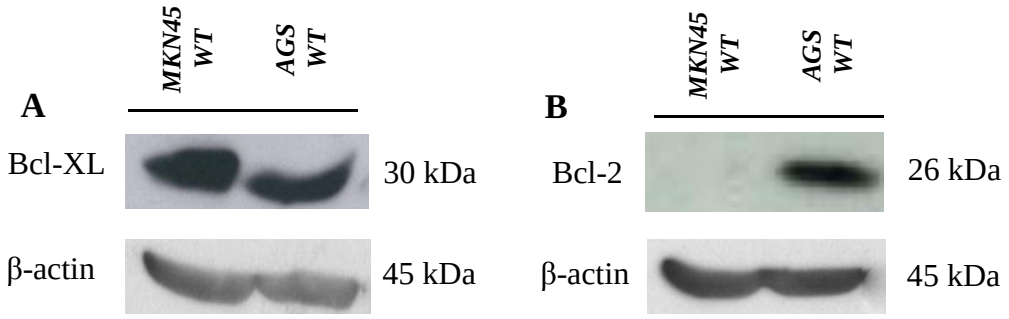

Figure 21: Western blot analysis of Bcl-XL and Bcl-2 proteins in MKN45 WT and AGS WT cell lines. (**A**) Analysis of the expression levels of the Bcl-XL protein (**B**) Analysis of the expression levels of the Bcl-2 protein. Equal loading was confirmed with β-actin immunoblotting.

The expression levels of the caspase-3 were also assessed, and as depicted in Figure 22, no differences in the expression levels were observed when comparing the MKN45 and AGS cell lines (Figure 22 (**A**)). However, immunoblot analysis of both full-length and cleaved forms of PARP did show differences in their levels of expression between both cell lines, with a higher expression of both forms observed for the MKN45 cells (Figure 22 (**B**)).

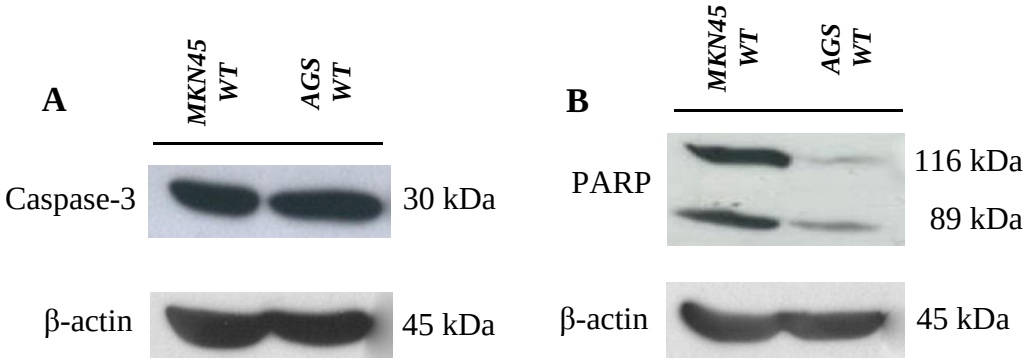

Figure 22: Western blot analysis of caspase-3 and PARP proteins in MKN45 WT and AGS WT cell lines. (**A**) Analysis of the expression levels

of the caspse-3 protein. (**B**) Analysis of the expression levels of the full-length and cleaved PARP protein. Equal loading was confirmed with β-actin immunoblotting.

4. Discussion

Reccent evidences suggest that gastric carcinomas, as many other tumours include a small population of cells with properties that are common to stem cells. This population of cancer stem cells (CSCs) has the ability to initiate and maintain tumour growth and to form metastasis (Bonet and Dick, 1997; Clarke et al., 2006; Reya et al., 2001).

During the last decade, several studies have been carried out using cell membrane markers such as CD44, CD24 and CD133, to identify and isolate the subpopulation of CSCs. These studies were performed in a variety of tumour models, such as breast (Al-Hajj et al., 2003), colon (O'Brien et al., 2007), melanoma (Schatton et al., 2008), liver (Yang et al., 2008), ovarian (Zhang et al., 2008), pancreas (Hermann et a., 2007), gastric (Takaishi et al., 2009), among others (reviewed in Schatton et al., 2009).

The main objectives of this study were the evaluation of the potential existence of a CSCs subpopulation in gastric cancer cell lines, as well as its influence on the resistance to chemotherapy drugs currently used in the treatment of gastric cancer, namely 5-fluorouracil (5-FU) and cisplatin (Kang et al., 2009; Kondo et al., 1996; Ohtsu et al., 1994; Rougier et al., 1994). In this work we also analyzed the expression levels of some proteins involved in apoptosis with the aim of assessing their possible involvement in the chemoresistance phenotype of cell lines and subpolations in study.

The models used in the present work were two gastric cancer cell lines, AGS and MKN45. Both cell lines were established from poorly differentiated gastric adenocarcinomas, present adherent cells and epithelial morphology. We used two cell lines in order to avoid cell line specific phenomena.

The resistance of the AGS and MKN45 gastric cancer cell lines and the AGS $CD44^+$ and $CD44^-$ subpopulations to the chemotherapy drugs was determinated by

estimating the GI50 value for each used drug. We performed the sulforhodamine B colorimetric assay (SRB), based upon the measurement of cellular protein content (Vichai and Kirtikara, 2006). This assay has been widely used for drug-toxicity testing against different types of cancerous and non-cancerous cell lines (Monks et al., 1991). Both cisplatin and 5-FU act by the inhibition of DNA synthesis (Gewirtz, 1999). So, it is important to take into account the right number of cells for each assay to ensure that these cells are in exponential phase during the experiments. The number of cells and the time of exposure to the drugs were based on the rate of cell division of each cell line. In previous studies performed by our group, the number of cells to use in each assay was $2x10^3$ cells/well for both AGS and MKN45 cell lines, and the time of treatment for each drug was 48 hours.

As already referred, the GI50 values of 5-FU and cisplatin in the AGS and MKN45 gastric cancer cell lines were evaluated. With that purpose, both cell lines were exposed to different concentrations of each drug for 48h (Figure 10 and 11). For the MKN45 cell line, the GI50 value of 5-FU was 5.42 ± 1.69 μM whereas for cisplatin a GI50 value of 4.69 ± 1.94 μM was obtained. In the case of the AGS cell line, when treated with 5-FU presented a growth inhibition of 50% at a concentration of 3.83 ± 0.88 μM μM, whereas the GI50 value of cisplatin was much higher reaching 18.97 ± 2.48 μM (Figure 12). These results demonstrated that the AGS cell line is much more resistant to cisplatin than the MKN45 cell line. For the 5-FU, both cell lines showed no significant differences with GI50 values of 5.42 ± 1.69 μM and 3.83 ± 0.88 μM for the MKN45 and AGS cell lines, respectively. Also, the AGS cell line is prone to be much more sensitive to the cytotoxic effects of 5-FU than to cisplatin (3.83 ± 0.88 μM for 5-FU and 18.97 ± 2.48 μM for cisplatin). The obtained results lead us to conclude that despite both cell lines have similar properties (adherent cells, epithelial morphology and

the both cell liens coming from poorly differentiated gastric carcinomas), they present different drug resistance profiles. These results support some studies showing that patients with tumours with similar histopathological classification can have different clinical drug-response (Furukawa et al., 1994). Moreover, the obtained cisplatin resistance profiles of both AGS and MKN45 cells are in accordance to what was observed in recent published results by Mutze et al (2010). In this study, the authors also stated that AGS cells were significantly more resistant to cisplatin than MKN45 cells with IC50 values of 18.5 µM and 7.1 µM for AGS and MKN45 cells respectively. The 5-FU resistance profile observed for the MKN45 cells in this study is similar to previously published results, describing a IC50 of 5-FU of 2.46 µM for this cell line (Cho et al, 2002).

As previously mentioned, a tumour is composed by a multitude of cancer heterogeneous cell populations. However, the therapeutic approaches used in the treatment of gastric cancer as well as all other cancers don´t consider this heterogeneity, with the currently available drugs being able to shrink tumours and metastasis but, most of them are unable to eradicate the tumours. Emerging data have shown that malignant tumours are composed of a small subset of distinct cancer cells that are responsible for tumour initiation and maintenance, known as cancer stem cells (CSCs). It is also thought that these cells are in the origin of the relapse and progression of a tumour, making the used chemotherapy ineffective, because these drugs don't eliminate the CSCs, which have been shown in several cancer types to be quite resistant to standard chemotherapy and radiation. (Lee et al., 2008, Reya et al., 2001; Schatton et al., 2009; Stockler et al., 2000). Considering this scenario, to achieve more effective treatments and a possible cure, the chemotherapy must be targeted to these small subsets of CSCs.

Thus, to demonstrate and analyze the possible involvement of CSCs in the failure of the chemotherapy treatment, many studies focus their objective in trying to isolate this subpopulation of cells. In the last year, Takaishi and his co-workers, identified CD44 as a marker of gastric CSCs, and, taking this into account, the CD44 glycoprotein was chosen as the potential marker of gastric CSCs during this work. Firstly, and before isolating the CSCs, the expression of the CD44 marker was analyzed in the gastric cancer cell lines by flow cytometry. As shown in figure 13, in the MKN45 cell line 99.15% of the population expressed CD44 at the cell surface (potential CSCs). On the other hand, the AGS cell line presents only 29.06% of potential CSCs (Figure 14). The verified results for MKN45 and AGS cell lines are similar to the ones observed by Takaishi et al. (2009), where in the MKN45 cell line more than 94% of the cells expressed the CD44 surface marker and in the AGS cell line between 10% and 50 % of the cells expressed the CD44 marker. So, despite having similar characteristics, not only these cell lines demonstrate different chemoresistance to the tested drugs, but also clearly show differences in the CD44 expression at the cell surface. Taking that into account, the AGS cell line was the one presenting better characteristics to allow the sorting of $CD44^-$ and $CD44^+$ subpopulations.

So and as mentioned before, two methods of magnetic separation were used: a direct (using anti-CD44 microbeads) and an indirect magnetic separation. This last separation was performed with anti-FITC microbeads, with the cells previously marked with the anti-CD44 - FITC antibody (the same antibody used to analyze the the expression of CD44 in the gastric cancer cell lines). These two approaches were needed to prevent variation on sorting specificity due to the antibody used. After the MACS, the GI50 value for each drug was also estimated for both the $CD44^-$ and $CD44^+$ subpopulations, with the purpose of evaluating a potential association of gastric CSCs

with the chemoresistance towards 5-FU and cisplatin. The analysis of the results depicted in figure 15, showed that $CD44^-$ and $CD44^+$ subpopulations, obtained by direct separation, have no significant statistical differences regarding 5-FU resistance. Also, no significant statistical differences were demonstrated between AGS WT population and the $CD44^+$ subpopulation and between both subpopulations ($CD44^+$ and $CD44^-$). On the other hand, after cisplatin exposure, the AGS WT population proved to be significantly more resistant than the $CD44^-$ subpopulation (*p*-value = 0.0239). The indirect method of magnetic separation provided the same results (Figure 16) as the ones obtained after the direct separation, except the comparison between the WT population and the $CD44^+$ subpopulation exposed to cisplatin, where the *p*-value was suggestive of a higher resistance of the WT cells (*p*-value = 0.0571). The results demonstrated that the WT population was generally more resistant to cisplatin when compared to the $CD44^-$ and $CD44^+$ subpopulations obtained by both methods of separation. These results are not consistent with previously published results. Several studies in myeloid leukemia, breast and pancreatic cancer, showed that the subpopulation of CSCs are indeed more resistant to chemotherapy drugs (Costello et al., 2000; Jordan et al., 2000; Lee et al., 2008; Reya et al., 2001; Schatton et al., 2009; Stockler et al., 2000).

The differences between our results and previous works might reflect the complexity of CSCs models and the impact of experimental strategies used. First, it is important to notice that the method used to isolate the $CD44^-$ and $CD44^+$ subpopulations is a method of enrichment (MACS) and not a method of purification (FACS). So the $CD44^-$ and $CD44^+$ subpopulations are not pure cell populations, but are, relatively to the WT population, subpopulations enriched in $CD44^-$ and $CD44^+$ cells, respectively. As a consequence, the GI50 values obtained may be affected by this heterogeneity. Secondly,

the antibodies used in the isolation of the $CD44^-$ and $CD44^+$ subpopulations also have to be taken into account (anti-CD44 microbeads, used in the direct magnetic separation and the anti-CD44 – FITC used in indirect separation). As mentioned before, the CD44 is a glycoprotein that has many isoforms generated by alternative splicing (Naot et al., 1997). Further diversification of the isoforms is generated by posttranslational modifications such as variable *N*- and *O*-linked glycosylations (Marhaba et al., 2008). Due to the existence of these different isoforms, it is possible that the antibodies used during this work, weren't able to effectively identify the epitope of the CD44 isoform present at the surface of AGS cells, affecting the sortig efficiency. Finally, another fact to consider is that this study was based on the isolation of the potential subpopulation of gastric CSCs from gastric cancer cell lines, and the process of establishing a cell line may involve a random and non-representative selection of the tumour cell population. So, this process can lead to a cell line that may not entirely mimic the initial composition of the primary tumour. Taking that into account, the AGS cell line might have been isolated from tumour areas that don't present stem cell population. Thus, all these aspects involving the biological model in study and the used methodology can be responsible for the absence of differences in drug resistance between the $CD44^-$ and $CD44^+$ subpopulations of the AGS cell line. It is also interesting to observe, that despite having 99.15% of $CD44^+$ cells, instead of the much smaller 29.06% seen in AGS cells, the MKN cell line displayed a significantly higher sensitivity to cisplatin. Usually a higher expression of CD44 is correlated to an increased resistance to chemotherapy. However, a recent study showed that in prostate cancer (aggressive PC-3M PCa cells), a stable re-expression of CD44 standard (CD44s) greatly reduced invasion, migration and tumour cell proliferation and enhanced chemosensitivity in vitro, suggesting a number of functional effects in PCa cells, for which CD44s functions as a tumour suppressor

(Yang et al, 2010). We can propose that if in gastric cancer CD44 might display similar functions as in prostate cancer, our chemoresistance profile observed in vitro is in agreement with the pro-chemosensitivity role played by CD44 in PCa. Indeed the cell line with the less expression of CD44 (AGS) was the most resistant to cisplatin. It is also important to take into account that the widely referred correlation between the expression of CD44 and increased drug resistance happens due to an *in vivo* constitutive interaction between hyaluronan and CD44. Such interaction was not mimicked in vitro in the present work and may have negatively affected the contribution of the expression of CD44 to a chemoresistance phenotype.

As demonstrated in figures 15 (**B**) and 16 (**B**), the WT population shows a higher level of resistance to cisplatin comparatively to the AGS $CD44^-$ and $CD44^+$ subpopulations. These results may arise from the magnetic separation process used in the isolation of the subpopulations. In the process of magnetic separation, cells are exposed to magnetic fields, which are known to induce hyperpolarization of the cell membrane and alter the transport properties of the plasma membrane (Seidl et al., 1999). Thus, the process of separation can be responsible for a potential alteration in the membrane of the $CD44^-$ and $CD44^+$ cells, due to the stress that these cells were subjected during this process. It may be the reason for the more cisplatin-sensitive phenotype of both isolated subpopulations when compared to the non-stressed WT population.

Additionally, the results showed that the $CD44^-$ was in fact the subpopulation that presented lower resistance to cisplatin, possibly due to the fact that this subpopulation was passed twice by the magnetic column (as described in materials and methods – 2.3.2), which can further stress the cell membrane. As seen in figures 15 (**A**) and 16 (**A**), the AGS $CD44^-$ and $CD44^+$ subpopulations were more resistant to 5-FU

than the WT population, contrarily to what was observed for cisplatin, where the WT cells showed higher resistance. The reason of the higher sensitivity of the WT cells to 5-FU when compared to the isolated subpopulations needs to be further investigated in the future. It is also important to note that the verified results were consistent for both of the methods of separation used. However, the drug resistance profiles verified for the AGS cells and respective $CD44^+$ and $CD44^-$ subpopulations still urge further confirmation in future studies due to the somewhat small differences observed. Perhaps an alternative more efficient and less aggressive method of separation could provide different results than the ones obtained in the present study. Very significant is the considerably higher resistance of the AGS cells towards cisplatin when compared to 5-FU.

As an analogue of uracil, 5-FU is incorporated intracellularly into the same DNA and RNA metabolic pathways as uracil. This incorporation of 5-FU is the essential mechanism of its cytotoxic effect. 5-FU is converted to several active metabolites which disrupt RNA synthesis and inhibit the action of thymidylate synthase (TS), a key enzyme of DNA synthesis. Given that both the transport of uracil through the cell membrane and its incorporation in several metabolic pathways are of crucial importance for mammalian cells, hence highly conserved and operative, and that the analogue form 5-FU is mistakenly recognized by these mechanisms as uracil, the intracellular accumulation of 5-FU is very effective allowing it to unleash its cytotoxic effects. Additionally, as a uracil analogue, 5-FU may be much less prone to an efflux out of the cell by ABC-transporters, normally overexpressed in cancer cells, especially when compared to the platinum-based cisplatin. The anti-tumour activity of cisplatin is generally due to its formation of DNA adducts which induce structural distortions in the DNA inhibiting its replication and transcription and, ultimately, inducing programmed cell death, or apoptosis (Trimmer and Essigmann, 1999; van Moorsel et al., 1999). As a

metal derived compound, and given that platinum isn't present in the wide variety of intracellular mechanisms of a mammalian cell and may be rapidly considered by the cell as a homeostasis-disturbing, its efflux is very likely to be much more effective than in 5-FU. Moreover, for the same reasons, the permeation of cisplatin through the membrane may be smaller than the one of 5-FU. These are all possibilities that may be behind the significantly higher resistance to cisplatin when compared to 5-FU displayed by AGS cells. For instance, Hyplat, a compound produced by cross-linking hyaluronan via its carboxylate groups with cisplatin at a high temperature, was more effective than cisplatin at slowing the growth of intraperitoneally inoculated A2780 ovarian cancer cells and improving survival by showing targeting ability to CD44 expressing cells, and superior pharmacokinetics and pharmacodynamics to free cisplatin inside the cell (Li and Howell, 2009).

Considering the results, it is important to understand and clarify the mechanism that is behind the differences in the resistance to 5-FU and cisplatin in both MKN45 and AGS cell lines and in the AGS respective AGS $CD44^-$ and $CD44^+$ subpopulations. A mechanism that can be responsible for the chemoresistance phenotype is a possible overexpression of anti-apoptotic or an underexpression of pro-apoptotic proteins in the MKN45 and AGS cell lines, as well as in the AGS $CD44^-$ and $CD44^+$ subpopulations. So, during this work, and as a parallel study, the expression of apoptosis-related proteins was analyzed in these cells. Apoptosis plays an important role in development, immunological competence, and homeostasis (Allan e Clarke, 2009; Boulars et al., 1999), and is critical in the pathogenesis of a variety of diseases including autoimmune disease, viral infection, neurodegenerative disorders and cancer (Kondo et al., 1998). Elucidation of the underlying regulatory mechanisms is relevant not only to disease etiology, but also to treatment. In particular, as cytotoxic therapies for cancer rely

heavily upon inducing apoptosis, a deeper understanding of apoptosis regulation in normal and neoplastic cells is important (Adams and Cory, 2007). By the assumption that the CSCs are in the origin of the resistance to chemotherapy, it is reasonable to analyze if the potential subpopulations of CSCs show differences in the expression levels of some proteins involved in apoptosis that may be involved in the chemoresistant phenotype often attributed to the CSCs. To accomplish this task some proteins involved in the apoptotic pathways were analyzed in the AGS cell line as well as in their $CD44^-$ and $CD44^+$ subpopulations, such as the Bcl-2, Bcl-XL, caspase-3 and PARP.

The Bcl-2 family of proteins plays a central role in the regulation of apoptotic cell death induced by a wide variety of stimuli (Reed, 1994; Reed, 1997). A member of the Bcl-2 related family of genes is the Bcl-XL protein (Kondo et al., 1998). Bcl-XL and the Bcl-2 proteins are key anti-apoptotic proteins (Minn et al., 1999; Yang et al., 1997), and their increased expression is involved in the development and progression of many tumours (Gautschi et al., 2001). As shown in figure 17, the immunoblotting for Bcl-XL expression did not reveal significant differences between the $CD44^-$ and $CD44^+$ subpolations and the WT cells, for both methods of magnetic separation (direct and indirect magnetic separation). Also, the Bcl-2 protein was expressed in similar levels in the AGS WT cells and the $CD44^-$ and $CD44^+$ subpolations, for both methods of separation (Figure 18), suggesting that both Bcl-XL and Bcl-2 weren't significantly contributing to the observed differences in chemoresistance profiles. Another protein whose expression pattern was evaluated was the caspase-3, using an antibody that recognizes the non-cleaved form. This protein has been identified as a key mediator of apoptosis in animal models, being an effector caspase that become active by cleavage (Broughton et al., 2010; Hamacher et al., 2008). In a similar manner as Bcl-XL and Bcl-

2, the analysis of the expression of the caspase-3 protein demonstrated, as shown in figure 19, that the levels of this protein were very similar between the $CD44^-$ and $CD44^+$ subpolations and the WT cells, whether the subpolations were isolated by indirect or by direct magnetic separation. This result also shows that the chemoresistant phenotypes of the different subpopulations studied are not directly related to the expression levels of this apoptotic protein. Another apoptotic protein investigated was poly (ADP-ribose) polymerase (PARP). This protein is involved in the repair of DNA and is constituted by 116kDa when in its full-length constitution. During apoptosis, PARP is cleaved, becoming inactive, by caspase-3 into a 89kDa fragment, which leads to DNA injury and subsequently to apoptotic cell death. So the PARP protein can be considerate as an apoptotic marker (Bhaskara et al., 2009; Boulares et al., 1999; Broughton et al., 2010; Endres et al., 1997; Namura et al., 1998). The antibody used in the immunoblot of this protein is capable of detecting both the cleaved and non-cleaved forms. As demonstrated in figure 20 (**A**), it was possible to observe that a higher expression of the PARP protein, in both forms, was visible in the $CD44^+$ subpolation when compared with the $CD44^-$ subpolation and WT population, being the differences more significant for the cleaved form. The $CD44^+$ subpolation thus present a higher amount of the form of the protein that leads to apoptosis. These results show that the population enriched in CSCs was the one expressing an increased amount the pro-apoptotic PARP form and are contrary to what has been described. On the other hand, the cells isolated by indirect magnetic separation, didn't show any differences in either PARP form expression between the $CD44^-$ and $CD44^+$ subpolations and the WT cells. These varying results regarding the PARP expression in subpolations sorted by two different cell sorting methods, raise the issue about the impact of the separation method on the expression profile of apoptotic proteins.

The expression levels of the same apoptosis-related proteins were also analyzed in the MKN45 WT and AGS WT cell lines. As verified in figure 21 (**A**), there was a higher expression of Bcl-XL in the MKN45 cell line when comparing to the AGS cell line. This result shows that the chemoresistant phenotype of the AGS cell line to cisplatin is not directly related to the expression of this anti-apoptotic protein. The same conclusion can be reached for the pro-apoptotic caspase-3, which didn't show differences in its expression levels between both cell lines (figure 22 (**A**)). On the other hand, the anti-apoptotic protein Bcl-2 is only significantly expressed in the AGS cell line and not in MKN45 cells (figure 21 (**B**)), suggesting that the increased cisplatin resistance of the AGS cell line may be related with a Bcl-2 overexpression. Previous studies have confirmed that overexpression of Bcl-2 in the cells of various malignant tumours could result in resistance against cell apoptosis induced by chemotherapeutic agents such as cisplatin (Rudin et al., 2003; Siervo-Sassi et al., 2003). As mentioned above, PARP weights 116kDa in its full-length anti-apoptotic form and, during apoptosis, is cleaved into a 89kDa fragment, unable to arrest apoptosis and a typical apoptosis marker. As demonstrated in figure 22 (**B**) a higher expression of both forms of PARP was observed for the MKN45 WT cells when compared to the AGS WT cells, with a slightly higher expression of the full-length form. These observations suggest that the expression of PARP cannot also be directly related to the enhanced cisplatin resistance of the AGS cell line. Considering the expression patterns of all these apoptosis-related proteins, we suggest that the chemoresistant phenotype demonstrated by the AGS cell line to cisplatin may not be the direct result of the presence of a subpopulation of CSCs, as well as of an overexpression of Bcl-XL and/or full-length PARP. Thus, it is extremely important to extend these studies in order to understand what mechanisms are responsible for this increased chemoresistance showed by the

AGS cell line, in order to further develop efficient therapies for gastric cancer in the future.

5. Closing remarks

5.1 Conclusions

The main goal of this work was to clarify the involvement of gastric cancer stem cells (CSCs) in gastric cancer resistance to currently available drugs and the identification of putative molecular mechanisms involved in chemoresistance of gastric CSCs.

Taking into account all the results obtained during this work, it was possible to conclude that, although the AGS and MKN45 cell lines have some similar phenotype properties, the AGS cell line displays a higher resistance to cisplatin than the MKN45 cell line, and, on the other hand, both cell lines showed a similar resistance to 5-FU. Given this difference, it is important to know and understand the mechanisms behind the chemoresistant phenotype of AGS cells.

The AGS and MKN45 cell lines presented a potential subpolation of CSCs ($CD44^+$), with the MKN45 cell line clearly showing a higher expression of CD44 than the AGS cell line. Interestingly, the cell line with the lower expression of CD44, the AGS, is the one displaying resistant higher resistance to cisplatin. After this observation and understanding the mechanism of action of the drug, it is extremely important to comprehend the interaction or relationship between chemoresistance and CD44.

The results lead us to suggest that the subpopulation enriched in CSCs ($CD44^+$) is not responsible for the cisplatin-resistant phenotype shown by the AGS cell line, since there was no difference in the resistance of the subpopulation enriched in gastric CSCs whether when treated with cisplatin or 5-FU. Our work also raises a question that it is imperative to debate: if CD44 really is a reliable marker of gastric CSCs? If indeed CD44 is a marker of gastric CSCs it is important to obtain as many as possible adequate antibodies for the different CD44 isoforms. Whatever the further studies with CD44, it

is urgent to find an alternative or set of markers capable of being used for identify gastric CSCs. The present study suggests that the subpopulations of potential CSCs ($CD44^+$) don't have significant differences in the expression levels of several proteins involved in apoptotic pathways, whether anti- (Bcl-2, Bcl-XL, PARP) or pro-apoptotic (caspase-3). In general, the drug resistance seems to be a result of other mechanisms than the overexpression of anti-apoptotic or under-expression of pro-apoptotic proteins. Only the anti-apoptotic protein Bcl-2 could apparently be correlated to the AGS resistance to cisplatin.

Finally, and given the results obtained for the expression of apoptotic proteins in cells sorted by direct and indirect MACS, further studies should be performed in order to evaluate the impact of isolation methods in CSCs phenotype.

Gastric cancer still isn't well understood, and many other studies will have to be carried out in order to comprehend the mechanisms leading to its development and chemoresistance. Only then, efficient chemotherapies would be developed, allowing the long-awaited decrease in the mortality of gastric cancer.

5.2 Future perspectives

With the aim of further advance the present work, several studies still need to be performed in the future. For instance, in order to obtain a more reliable potential subpopulation of gastric CSCs, it might be useful to use samples of primary tumours. This way, it will be possible to mimic more realistically the constitution of the tumour subpolations. Also very important is the confirmation of CD44 as a specific marker of gastric CSCs, which is yet to be achieved after this study. If CD44 indeed proves to be a reliable marker of gastric CSCs, future investigations must be directed to develop tolls

(antibodies) that detect the different isoforms of CD44 in gastric cancer. The development of specific antibodies to the different CD44 isoforms will allow the sorting of a reliable subpopulation enriched in stem cells but, more importantly, also the use of CD44 as a target of efficient chemotherapy. Additionally, confirmation by flow cytometry of the much higher expression of CD44 in the CSCs-enriched subpopulation after cell separation also seems to be a step to be taken in future studies so that biased results can be avoided. In order to obtain a high pure subpopulation of CSCs, a better method of separation, such as FACS, might be needed in future studies, instead of an enrichment method, as MACS. This way, the influence of CSCs in the resistance towards chemotherapy will be more correctly evaluated, which will also benefit the development of more efficient treatments. A detailed and comparative analysis of the various different signaling pathways intimately involved in either cell survival or cell proliferation in both $CD44^-$ and $CD44^+$ subpolations in future studies, hopefully will identify new and very useful targets that can also be used as therapeutic targets.

The resistance of CSCs to chemotherapy has been described as being in part due to an overexpression and increased activity of drug efflux pumps, such as MDR1 and ABCG2 (Klonisch et al., 2008), so, in the future it will be important to analyze their expression and activity in the subpopulation of gastric CSCs, and also, to understand which drugs commonly used in chemotherapy are more prone to be effluxed by these proteins.

In conclusion, further investigation still needs to be performed, in order to develop more efficient chemotherapies against gastric cancer, and many doubts still surround CD44 as a good target for such treatments. Gastric CSCs are yet to be specifically targeted by the presently developed drugs, and we can only hope that future studies will help to decrease the burden worldwide of gastric cancer.

6. References

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