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Gastric Cancer Immunotherapy: An Overview

Fabrizio Romano[*], Fabio Uggeri, Luca Nespoli, Luca Gianotti, Mattia Garancini, Matteo Maternini, Angelo Nespoli, Franco Uggeri

Department of Surgery, San Gerardo Hospital, University of Milano-Bicocca, Monza, Italy.
Email: [*]fabrizio.romano@unimib.it, fabriziorom@hotmail.com

ABSTRACT

Gastric adenocarcinoma is the third most common cancer and the second most common cause of death due to cancer worldwide. Surgery is still the major prognostic factor for gastric cancer. Patients who could not be resected have a poor prognosis with survival ranging from 3 to 11 months. There is evidence that surgical operations can cause a variety of immunological disturbances in man both *in vivo* and *in vitro*. The postoperative changes in the systemic immune response are proportional to the degree of surgical trauma leading to a generalized state of immunosuppression, which is implicated in the development of septic complications and provided a "fertile soil" for tumor cell metastasis. Immunotherapy may be a potentially promising alternative strategy for gastric cancer. In early clinical trials, systemic immunotherapy included both active vaccination directed against defined tumor-associated antigens expressed in gastric carcinoma cells and passive administration of IL-2 with some evidence of regression of metastatic gastric cancer. Other studies have applied immunotherapy in the adjuvant setting with equally promising results. For example, OK-432, a streptococcal preparation, demonstrated marginal improvement in survival for patients with stage III gastric cancer and a meta-analysis of centrally randomized controlled clinical trials indicated a significant survival benefit with combination OK-432 and chemotherapy compared to chemotherapy alone ($p < 0.05$). Additional data suggesting a biological and clinical benefit of subcutaneous, preoperative administration of low-dose IL-2 in colon cancer encouraged us to evaluate low-dose IL-2 therapy in the neoadjuvant setting for patients with gastric adenocarcinoma who undergo surgery and to evaluate its effects on systemic and tumor infiltrating lymphocyte numbers. We also sought to determine if neoadjuvant low-dose IL-2 could influence the clinical outcome for patients undergoing gastric resection for cancer. We report the biological, histological and clinical results with the full accrual of patients and a median follow-up of 51 months.

Keywords: Gastric Cancer; Immunotherapy; IL-2

1. Introduction

1.1. Incidence and Risk Factors

A global cancer statistic made in 2002 [1] refers an incidence of gastric cancer of 8.6% worldwide (934,000 new cases per year in 2002), which poses it in fourth place behind cancers of the lung, breast, colon and rectum. It is still the second most common cause of death from cancer worldwide (700,000 deaths annually). Almost two-thirds of the cases occur in developing countries and 42% in China alone. The geographical distribution of stomach cancer has wide international variations; high-risk areas include East Asia (China, Japan), Eastern Europe, and parts of Central and South America. Survival for stomach

cancer is moderately good only in Japan (52%), where mass screening has been practiced since the 1960s. Survival is also relatively high in North America, possibly due to early diagnosis following a greater number of endoscopic examinations performed for gastric disorders.

The evidence linking *Helicobacter pylori* infection to cancer of the stomach was considered sufficient by IARC [2] to classify this bacterium as carcinogenic in humans. Its action is probably indirect by provoking gastritis, gastric atrophy, metaplasia, and dysplasia. *Helicobacter pylori* has an high prevalence worldwide with a relatively small variation, which suggests than other factors are of major importance in gastric cancer pathogenesis. Diet certainly plays an important role: preserved salted foods are high risky. Tobacco smoking has also been clearly accepted as increasing the risk of stomach cancer [3].

[*]Corresponding author.

Even if the incidence of gastric cancer is decreasing worldwide, recent reports referred that tumors of the cardia are on the rise, especially among males and notably in the UK, Ireland, Northern Europe, Australia and New Zealand, China, and North America [4-8]. The rate at which the incidence of proximal stomach cancers has risen exceeds that of any other cancer [8,9]. The increase in tumors of the cardia is taking place principally in countries with relatively low overall rates of gastric cancer. Proximal tumors are associated with higher social class. *Helicobacter pylori* in these cases seem to play a smaller role.

1.2. Staging, Surgical Resection and Lymphnodes Dissection

For those patients with gastric cancer who undergo surgery, the factors that affect prognosis are the location of the tumor, the gross pathologic type and the TNM classification [8]. There are currently 2 major staging systems for patients with gastric cancer. The Japanese use an elaborate system that focuses on a complex anatomic nodal classification scheme. Western countries have adopted a system developed jointly by the American Joint Committee on Cancer (AJCC) and the International Union Against Cancer (UICC) [10,11]. In 2002, the AJCC and UICC agreed on a common staging system changing from a nodal staging system based on distance from the primary tumor to a staging system based on the number of positive nodes. Approximately one-third of gastric cancer patients have stage I or II disease at the time of diagnosis. One-quarter have stage III disease, and the remaining 40% stage IV disease [12]. Estimated adjusted survival 5-year survival after surgery is 82.9% for stage I, 62.8% for stage II, 17.8% for stage III and 3.3% for stage IV [13]. One-year survival rates are 50% in stage IIIA and B disease, and <25% in stage IV disease. The development of endoscopic techniques has improved the proportion of gastric cancers detected at an early stage.

Surgical resection remains the primary treatment for gastric cancer. Patients who could not be resected have a poor prognosis with survival ranging from 3 to 11 months. Poor prognosis may result by the fact that when the symptoms occur, the cancer has already spread and only a few percentages of patients are eligible for surgery. The aim of surgery is to remove completely the grossly visible tumor and obtain histologically free surgical margins (R0 resection). Only a complete-R0-resection can improve prognosis. The extension of gastric resection depends on the location of the tumor. In our experience total gastrectomy is preferred to partial gastrectomy when tumors are localized to the cardia. Reconstruction of digestive continuity is then realized by Billroth II termino-lateral gastro-digiunal anastomosis in the case of partial gastrectomy and by termino-lateral esophago-di-giunal anastomosis with digiunal loop in case of total gastrectomy.

Lymphnodes dissection is a debated issue in management of gastric cancer. The question of whether the use of a large limphonodes dissection (D2-D4 resection) increases cure rate when compared with limited lymphadenectomy of the perigastric nodes (D1 resection) remains open. A factor which compromises any study comparing survival in patients treated with extensive versus standard lymph node dissections is the "stage migration effect". This is based on the fact that more lymph nodes examined, the greater the probability of positive nodes. For example, some authors reported that up to 75% of patients classified as stage IIIB on D1 dissection were reclassified as stage IV on D2 dissection [14]. The best method to assess effect of surgery on survival is to compare intermediate stage patients (T3 tumors) regardless of the nodal status. This group would be most likely to benefit from extended lymph node resections. For example, the Memorial Sloan-Kettering Cancer Center reviewed their results in 774 patients who had undergone curative gastric resections [15]. They reported survival rates in patients with T3 tumors treated with D2 and D1 dissections of 54% and 39%, respectively. They concluded that D2 dissection offered a survival advantage for T3 tumors. Other large randomized studies have compared D1 and D2 resections in patients with gastric cancer and had reached nearly identical conclusions that D2 resections offered no survival advantage and were accomplished at the expense of greater operative morbidity and mortality [16,17]. Recently the Cochrane Review concluded that modified D2 resections can be and currently are being performed with much lower operative morbidity and mortality [18].

1.3. Adjunctive Therapies to Surgery

Because of the poor outcomes of surgery for gastric cancer, there has been much interest in adjunctive therapies that, when used in addition to surgical removal of the primary tumor, may improve survival [19-22]. Adjuvant cytotoxic chemotherapy is successful in few gastrointestinal cancers [22] and many phase 3 clinical trials [19,20] have explored this approach in gastric cancer. However, the survival benefit gained from the use of adjuvant chemotherapy in gastric adenocarcinoma seems to be not clinically significant [19,20] and for this reason adjuvant chemotherapy has not become part of the standard of care in gastric cancer.

Adjuvant chemotherapy rationale is a high risk of peritoneal and/or haepatic recurrence of gastric cancer. Drugs usually employed are 5-Fluorouracil (5-FU), Mitomycin-C, Cisplatin, Adriamycin, and Methyl-CCNU. Many authors report poor results in survival rate and, contrary to oldest trials, a recent prospective controlled

study [23] did not demonstrate any improvement in survival by the administration after surgery of an association of 5-FU + cisplatin versus no treatment.

Intra-peritoneal chemotherapy is an alternative to the systemic way, essentially in prevention of peritoneal diffusion and seeding. Until now there were 5 controlled trials [24-28] about intra-peritoneal chemotherapy. In stage III cancer patients there was a significant difference in 5-year survival rate between those treated with early post-operative intra-peritoneal chemotherapy and those who underwent surgery alone [28]. This technique has high morbidity and mortality, it could be interesting in prevention of carcinomatosis but in selected patients.

The efficacy of radiotherapy has not been demonstrated in gastric cancer patients. Most of the trials associate radiotherapy and chemotherapy.

Other than these classical non-surgical interventions, immunotherapy is a well demonstrated approach for few cancers and may also be a potentially promising alternative strategy for gastric cancer. The benefit of immunotherapy is the specificity. It distinguishes the biological differences between normal and malignant cells to exert tumor cell death in a precise manner.

2. Review Immunotherapy and Gastric Cancer

2.1. Tumor Immunology and Tumor Escape

There is evidence that cancer to grow has to deal with immune system. This evidence has been demonstrated scientifically since early' 90, when tumor antigens were identified and characterized and noted to be recognized by cytotoxic T cells [29]. These proteins, produced by mutated DNA segments, are potentially immunogenic and could be recognized by immune system as antigens to reject. Immune responses against tumor antigens reflect immune responses against normal "self" tissue antigens, with some important and significant features that distinguish the tumoral pattern from the normal one. Tumors differ fundamentally from their normal cell counterparts in antigenic composition and biologic behaviour [30]. The molecular hallmark of carcinogenesis is a genetic instability [31]. This means that new antigens are constantly generated in tumors as they develop and progress. The normal antigens pattern instead maintains a stable profile. During tumorigenesis, hundreds of genes that are usually inactive in the normal tissue or expressed at relatively low levels are dramatically activated, raising the concentration of encoded proteins and affecting antigenicity [30].

Another important characteristic that qualify the malignancy of each tumor and directly affect morbidity and mortality is the ability of transformed malignant cells to invade across natural tissue barriers and to metastasize.

Both these features are never seen in normal tissues or benign tumors and they are associated with disruption of tissue architecture. At a biological level the most important consequence of tissue disruption, even when caused by non-infectious mechanisms, is the elaboration of pro-inflammatory signals [30]. These signals, generally in the form of cytokines and chemokines, are central initiators of both innate and adaptive immune responses.

These two characteristics: the ability to produce antigens and to elicit a pro-inflammatory cascade, demonstrate cancers' immunogenicity. The consequence of these findings is that tumor, to grow, needs to inhibit host's effective immune response.

In the past years many were the attempt to identify and characterize the mechanisms of tumor growth against host's immune system. One of the initial theory about it, which was firstly rejected but now reconsidered, is called: "immunological surveillance" [32,33]. This hypothesis consists in the fact that tumors can arise with the same frequency of infections caused by pathogens, and that immune system constantly suppresses them, recognizing tumor associated antigens. Those tumors clinically progressing are not eliminated by immune system because they are able to develop mechanism of immune escape or resistance.

In the past decades many attempts have been made to identified and characterized possible mechanisms used by tumors to escape immune surveillance [34,35] and these can be summarized in the down regulation of MHC class I pathway, the lack of co-stimulatory function, the natural selection of poorly immunogenic tumor cells, the loss of tumor antigens, the defective "death receptors" signal, the suppression of immune response by tumor produced cytokines, the apoptosis of activated T cells and, recently introduced, the regulatory function of $CD4^+CD25^+$ T cells (regulatory T cells). No one of these mechanisms could explain alone tumor's growth in an immunocompetent host.

- The down regulation of the antigen processing MHC class I pathway has been documented in a large variety of human tumors, particularly breast cancer, prostate cancer and lung cancer [36-52]. Down regulating MHC class I pathway, antigens presenting cells are not able to process tumoral antigens and present them to T lymphocytes, leading to an ineffective cellular mediated immune response.

- To build an effective immune response, the immune system needs the presence of co-stimulatory molecules (e.g.: B7-1, B7-2). Lack of expression of these molecules by tumor cells will not lead to dendritic cells activation and maturation after recognition of tumor antigens, leading to T cell anergy [53]. In an experimental setting, insertion of genes encoding B7-1, B7-2 or both into tumors generally increases

the immunogenicity of those tumors but does not necessarily lead to cancer regression [54].

- Genomic instability, the main property of all tumors gives rise to genetic diversity [35]. The survival of cancer cells depends on natural selection, a passive process by which an organism has a survival advantage simply by possessing certain specific genes. Genomic instability creates a vast repertoire of tumor cells that will be selected according to environmental factors.

- The exact mechanisms that control the down-regulation of tumor antigens may be facilitated by "epitope immunodominance" [55]. The "immunodominance" consist in a preferential immunodetection of one or a few epitopes among many expressed on a given target. The parental tumor cells that carry the immunodominant epitope lead to a confined immune response, diverting attention from the tumor variants. Once the parental cells are eliminated, a new hierarchy is established among the variant subpopulations, and formerly immunorecessive epitopes become dominant [55].

- Two death receptor ligands that play a role in immune surveillance against tumor development are Fas ligand (FasL) and TRAIL [56-59]. These molecules transmit an apoptotic signal via the caspase cascade, through cytoplasmic sequences called "death domains". Loss of the gene encoding Fas has been identified in multiple myeloma [60], non-Hodgkin's lymphoma [61] and melanoma [62]. In addition, mutations of the genes in the proximal pathways downstream of Fas signaling have been also found in NSCLC and lymphoma [63,64].

- Activation or inhibition of T cells also depends on the presence or absence of cytokines in their immediate microenvironment. Tumor cells produce a variety of cytokines and chemokines that can suppress immune cells' function. Vascular endothelial growth factor (VEGF) is a cytokine secreted by most tumors [65] and *in vitro* studies show it inhibits dendritic cells differentiation and maturation [66]. In patients with gastric [67], lung, head and neck, and breast cancers [68], it has been noted a decrease in the function and number of mature dendritic cells, associated with increased plasma concentrations of VEGF. This fact was associated with a poor prognosis in gastric cancer patients. Also Interleukin-10 (IL-10) is a cytokine frequently detected in the serum of patients with cancer. IL-10 has an inhibitory effect on dendritic cells differentiation, maturation [69], antigen presentation, Interleukin 12 (IL-12) production and induction of T helper type 1 (Th1) responses *in vivo* [70,71]. It also enhances spontaneous dendritic cells apoptosis [72], and protects tumor cells from cytotoxic T cells func-

tion by down-regulation of HLA classes I and II and ICAM-1 (intercellular adhesion molecule 1) [73].

- Recently, a subpopulation of thymus-derived CD4^{+} T cells, displaying high levels of CD25, has been described to suppress the function of self-reactive T cells in the periphery [74-76]. These so-called regulatory T cells express all three subunits of the IL-2R but do not secrete IL-2 and, therefore, depend on exogenous IL-2 for survival and function. This has been demonstrated through *in vitro* mixed culture assays in which CD4^{+}CD25^{+} regulatory T cells mediate suppression of CD4^{+}CD25^{−} cells only in the presence of IL-2 [77]. Furthermore, IL-2 knockout mice, characterized as having significant impairment in regulatory T cells, develop severe autoimmunity that can be reversed by reconstitution of that pattern of cells from normal mice. The possibility that CD4^{+}CD25^{+} cells may influence patients with cancer has been suggested by the increased numbers of regulatory T cells demonstrated in a variety of human cancers, such as pancreas and breast adenocarcinoma, lung, gastric, esophageal, hepatocellular, ovarian cancer, melanoma and renal cell cercinoma [78-84].

2.2. Immunity and Surgery

Surgery is still the major prognostic factor for many solid cancers, such as the gastric one. There is evidence that surgical operations can cause a variety of immunological disturbances in man both *in vivo* and *in vitro* [85]. During the early postoperative course this may define a state of impaired defence and increased susceptibility to infection, septic complications and metastatic diffusion [86-88], in particular in patients with politraumatic event, burn injury and cancer. During the past decade, experimental and clinical research has focused on the understanding of the pathophysiology of this immuno-inflammatory dysfunction.

Primarily, surgical trauma induces an acute phase response, which is capable of controlling tissue damage, killing infective organisms, and inducing repair processes in order to restore normal host function [88-90]. The acute phase response is initiated locally at the site of the surgical trauma by macrophages and monocytes, which release pro-inflammatory cytokines, in particular tumour necrosis factor alpha (TNF-α) and interleukin-1 (IL-1) [91]. Studies refer that excessive release of TNF-α and IL-1, lead to increased mortality and an increased risk of postoperatory development of adult respiratory distress syndrome (ARDS) and multiple organ failure (MOF) [92]. TNF-α and IL-1 stimulate the production of other cytokines, such as interleukin-6 (IL-6), which peaks at 4 - 48 hours after surgery [93-94]. The release of IL-6 seems also to depend on the extent of tissue trauma [95]. In fact several studies demonstrates that there is a greater

degree of IL-6 elevation after abdominal aortic and colorectal surgery than after hip replacement [95] and that there are lower IL-6 levels after laparoscopic procedures than after open operating procedures, including cholecystectomy and small bowel and colonic resections [96-100]. IL-6 stimulates neutrophils to produce acute phase proteins, such as C-reactive peptide, alpha-2 macroglobulin, which are involved in non-specific and specific immunity as inflammatory mediators, scavengers and protease inhibitors [91]. Other than this, IL-6 plays a major role in the proliferation of PMN progenitors in the bone marrow, and mechanical trauma has been shown to induce an early increase in circulating imamture PMNs [101]. After open cholecystectomy, which is associated with higher IL-6 levels, PMN superoxide anion release and chemotaxis are increased, in comparison with the less traumatic laparoscopic approach [102]. IL-6 is also an important immunoregulatory cytokine [103]. IL-6-triggered acute phase response, in fact, induces macrophages to release prostaglandin E2 (PGE2) [90], which is probably the most powerful endogenous immune suppressant. PGE2 inhibits T-cell mitogenesis, interleukin-2 (IL-2) production, and IL-2 receptor (IL-2r) expression [104]. Stimulating interleukin-4 (IL-4) action, PGE2 also induces the release of IL-10 [105]. IL-6 is capable of downregulating pro-inflammatory cytokines and, additionally, of exerting profound anti-inflammatory actions, resulting in a dramatic cytokine misbalance, which is clinically reflected by a compensatory anti-inflammatory response syndrome [106]. Thus, as a final effect, surgical trauma-induced immunosuppression, which is characterized by low levels of pro-inflammatory TNF-α, IL-1, IL-12, and interferon gamma (IFN-γ) but markedly elevated levels of anti-inflammatory IL-6, IL-10, and IL-1 [103,106,107]. These results are further confirmed by studies that demonstrate that a reduction of surgical trauma by the use of a minimally invasive laparoscopic approach significantly reduces the increased serum levels of IL-6 and IL-10, and restores the decreased IFN-γ, TNF-α, and IL-2 production by T cells [100,108].

Moreover, cell-mediated immunity is based on an effective interaction between macrophages and T cells. During trauma or sepsis, macrophages and monocytes are triggered to produce and release PGE2, which initiates a sequence of events, resulting in T cells inhibition [109]. The process result also in a deactivation of monocytes, which is characterized by a reduced human leukocyte antigen (HLA-DR) receptor expression, a loss of antigen-presenting capacity, and a pronounced reduction in their ability to produce TNF-α [110]. This monocytic deactivation involves IL-10, because a neutralizing antibody against IL-10 has been shown to restore significantly the LPS-induced pro-inflammatory cytokine production [111]. Apart from IL-10, the anti-inflammatory

transforming growth factor beta (TGF-β) may additionally contribute to the surgical trauma-induced monocyte deactivation. Haemorrhage, and also major surgical trauma, such as thoraco-abdominal aortic aneurysm repair, induces a significant increase in circulating TGF-β [112, 113], which is associated with a marked depression of macrophage antigen presentation [112].

The innate cellular immune system seems to be inactivated by surgical trauma too. In fact, major surgical interventions are associated with a significant decrease in total systemic lymphocyte counts, including both CD4$^+$ and CD8$^+$ cells [114]. This lymphocyte depression has been shown to correlate with the duration of the surgical procedure, and the volume of blood loss, however, was not associated with the extension of the trauma, the age of the patient, or the type of intensive care intervention [114]. The cause of the fall in lymphocyte count due to surgical trauma may involve a dysregulated expression of apoptotic death and survival factors. A study demonstrated an increase CD4$^+$ and CD8$^+$ T cells' apoptosis 24 hours after surgery than before the intervention, associated with a downregulation of anti-apoptotic factors such as Bcl-2 [115]. The impact of this immune dysfunction was underlined by the result that the rate of apoptotic CD8$^+$ cells significantly correlated with the manifestation of infectious complications during the postoperative course [115]. Also this post-surgery increase of apoptotic CD4$^+$ and CD8$^+$ T cells' death is associated with the elevated levels of IL-10 [116].

Other studies demonstrated that modulation of T-helper lymphocytes is also involved in the development of immunosuppression after surgical trauma. Th cells can be subdivided into two functionally distinct subsets: Th1 and Th2 cells. Th1 cells may support an inflammatory response by producing IL-2, IL-12, and INF-γ, while Th2 cells act as anti-inflammatory agents by secreting IL-4, IL-5, IL-6, IL-10, and IL-13. After major surgery the cytokine secretion of T cells obtained from peripheral blood mononuclear cells (PBMCs), stimulated by crosslinking of CD3 and CD28, was reduced for IL-2, IFN-γ, and TNF-α, which are associated with the Th1 phenotype [89]. In contrast, the anti-inflammatory cytokine IL-10 was selectively elevated during the late postoperative course, indicating a shift of the Th1/Th2 balance towards a Th2 response [89]. That this deregulation of T lymphocyte function is induced by surgical trauma is confirmed by the results of another study, which demonstrates that minimization of the trauma by laparoscopic surgery results in restoration of an adequate Th1/Th2 and pro-inflammatory/anti-inflammatory cytokine balance [108].

2.3. Cancer Immunotherapy

One immediate goal of research in cancer immunology is

the development of methods to enhance a host's effective immune response against tumor. Many are the treatments based on a restoration of immune system. We call active immunization the attempt to train the patient's immune system to recognize tumor cells as targets to be destroyed. This could be obtained by the administration of tumoral antigens presented by dendritic cells, the so called "anti-tumor vaccine", or by autologous tumoral cells genetically modified in production cytokines. Another way to immunizate cancer patients is through the administration of therapeutic antibodies or cytokines, in which case the patient's immune system is recruited to destroy tumor cells by the therapeutic antibodies and cytokines (passive immunization).

Antibodies are a key component of the adaptive immune response, playing a central role in both in the recognition of foreign antigens and the stimulation of an immune response. The advent of monoclonal antibody technology has made it possible to raise antibodies against specific antigens such as the unusual antigens that are presented on the surfaces of tumors. While antibodies targeted to disease-causing antigens can be effective under certain circumstances, in many cases, their efficacy may be limited by other factors. In the case of cancer tumors, the microenvironment is immunosuppressive, allowing even those tumors that present unusual antigens to survive and flourish in spite of the immune response generated by the cancer patient, against his or her own tumor tissue.

Cytokines, such as IL-2, also play a key role in modulating the immune response, and have been tried in conjunction with antibodies in order to generate an even more devastating immune response against the tumor.

Vaccination can stimulate humoral, cellular and innate immune systems to generate various effector molecules, like antibody, cytotoxic T cells, cytokines etc. This is an antigen specific immunotherapy, in which the immune system can be stimulated actively by antigen based tumor vaccine to kill only those tumor cells, having expression of the particular tumor associated antigen.

2.4. Gastric Cancer Immunotherapy

Immunotherapy may be a potentially promising alternative strategy for gastric cancer. In early clinical trials, systemic immunotherapy included both active vaccination directed against defined tumor-associated antigens expressed in gastric carcinoma cells [117] and passive administration of IL-2 with some evidence of regression of metastatic gastric cancer [118,119]. Other studies have applied immunotherapy in the adjuvant setting with equally promising results. Adjuvant immunotherapy is expected to exert a synergistic effect with surgical resection. For example: OK-432, a streptococcal preparation, which is one of the most commonly used non-specific

enhancer of immune system, demonstrated marginal improvement in survival for patients with stage III gastric cancer [120]. Administration of OK-432 has been reported to induce the release of various cytokines, such as TNF-α, INF-β, IL-8, IL-12, etc., and has also been shown to enhance cytotoxicity against autologous cells. Furthermore it induces augmentation of perforine and the upregulation of FAS ligand, promoting anti-tumor necrotic and apoptotic effects by natural killer cells (NK) and lymphocytes activated killers (LAK). OK-432 has been initially administered in a neoadjuvant setting with preoperative endoscopical intratumoral injection to stimulate an increase of tumor infiltrating lymphocytes (TILs), which are a well-known prognostic factor in gastric cancer patients [121]. In OK-432 treated patients, TILs were moderately present in 40% and markedly present in 33% of cases, compared to 31% and 0% of cases respectively in the control group. A recent study demonstrate that OK-432 significantly increase the 5- and 10-year survival rates of patients with stage IIIA + IIIB gastric adenocarcinoma [120]. OK-432 was most effective in prolonging survival of patients who had both positive TILs and lymph node metastases. In fact, in TILs positive patients a significant decrease in metastatic lymph node frequency and in the number of lymph node micrometastatic foci was noted. It probably acts partly by eliminating micro-metastatic foci in lymph nodes. Different studies employed adjuvant OK-432 in a subcutaneous route of administration. In a recent study it has been showed a difference in overall survival between OK-432 and control groups, with a reduction of 13% in the 3-year death rate [121,122]. A meta-analysis of centrally randomized controlled clinical trials indicated a significant survival benefit with combination of OK-432 and chemotherapy compared to chemotherapy alone ($p < 0.05$) as shown in **Table 1** [123]. PSK (polysaccaride K) seems to achieve comparable results in an adjiuvant setting. Because of OK-432 and PSK have similar immunomodulatory effects, but are not necessarily effective as monotherapy against gastric cancer, the benefits of immuno-chemo-therapy after curative resection of gastric cancer may be caused by the restoration of immunity in patients who have immunosuppression because of surgery and subsequent chemotherapy. Recent advances in molecular biology suggest that immunomodulation by OK-432 may sensitize peripheral blood lymphocytes (PBL) to OK-432, leading to the subsequent activation and proliferation of cytotoxic effect cells.

Recent studies on gastric cancer demonstrated that gastrin-17 (G17), the predominant form of circulating gastrine hormone, is a tumor growth factor, with proangiogenic and antiapoptotic effects (**Figure 1**), and gastric cancer cells overexpress gastrin genes. G17DT is a therapeutic immunogen which elicit specific and high-affinity antigastrin antibodies to bind G17, thus preventing

Table 1. A meta-analysis of centrally randomized controlled clinical trials.

No.	Study	Interventions	Participants No.	Excluded population (%)	Analyzed cases	Male (%)	Stage III over (%)	Five year survival rate (%) death	Death	p-value
1	Niimoto et al. (SA G-Kyushu)	A: Mitomycin C(MMC) 20 mg i.v day 1, futrafal (FT) 00 mg/day for 1 year B: MMC 20 mg i.v day 1, PSK 3 g/day and FT 600 mg/day orally for 1 year	256	22.0	199			58.5	83	0.004
		B: MMC 20 mg i.v day 1, PSK 3 g/day and FT 600 mg/day orally for 1 year	264	28	191			71.7	54	....
2	Kondo et al. (SACG-Chubu)	A: FT 600 - 800 mg/day orally for 3 months with 2 months rest	208	23.6	159			63.6	58	0.135
		B: FT 600 - 800 mg/day orally for 3 months and PSK 3 g/day during 2 months rest	198	26.8	145			71.3	42	....
3	Kondo et al. (TGOG)	A: Carbazilquinone (CQ) 2 mg/m² I.V on day 0, 8 15 in each course	75	34.7	49	59.2	40.7	47.1	27	0.690
		B: CQ 2 mg/m² i.v on day 0, 8, 15 amd PSK 2 mg/m² orally from day 15 for 4 weeks	65	27.7	47	63.2	65.3	45.3	27	....
4	Ogawa et al. (KGSG)	A: MMC 0.4 mg/kg i.v day 0 and 0.2 mg/kg day 1, HCFU 400 mg/day orally for 1 year	69	20.2	55		21.0	81.1	9	0.810
		B: MMC 0.4 mg/kg iv day 0 and 0.2 mg/kg day 1, HCFU 400 mg/day and PSK 3 g/day orally for 1 year	70	20.0	56		41.0	80.2	11	....
5	Nakazato et al. (SIP)	A: MMC 6 mg/m² i.v day 0 and 1, fluorouracil (FU) 150 mg/day orally for 4 weeks with 4 weeks rest	129	0.0	129	66.7	65.1	60.0	52	0.044
		B: MMC 6 mg/m² i.v day 0 and 1, FU 150 mg/day orally for 4 weeks and PSK 3 g/day orally for 4 weeks	124	0.0	124	66.9	64.5	73.0	33	....
6	JFMTC-1	A: MMC 20 mg bolus day 0 and 1, tegafur 600 mg/day orally for 8 months	1.897	20.1	1.509	63.9	65.8	60.2	620	0.500
		B: MMC 20 mg bolus day 0 and 1, tegafur 600 mg/day orally and PSK 3 g/day for 8 months	1.942	16.7	1.601	61.8	66.8	61.2	638	....
		C: MMC 20 mg bolus day 0 and 1, tegafur 600 mg/day orally and OK 432 totally 100 KE for 8 months	1.907	18.5	1.543	63.8	69.1	58.7	660	....
		D: MMC 20 mg bolus day 0 and 1, tegafur 600 mg/day orally, OK 432 totally 100 KE and PSK 3 g/day for 8 months	1.891	17.8	1.544	65.7	68.5	60.2	633	....
7	JFMTC-5	A: Tegafur 600 mg/day orally	222	2.3	217	65.9	53.0	47.0	115	0.350
		B: Tegafur 600 mg/day orally and PSK 3 g/day	220	2.5	215	60.7	51.2	52.8	101	....
8	JFMTC-11	A: Surgery alone	114	1.8	112	65.8	8.7	83.3	20	0.769
		B: PSK 3 g/day orally for 6 months	114	0.0	114	67.0	5.4	84.8	19	....

SACG: The Co-Operative Study Group of Surgical Adjuvant Chemotherapy for Gastric Cancer; TGOG: Tokai Gastrointestinal Oncology Group; KGSG: Kunamoto Gastrointestinal Immunochemotherapy Study Group; SIP: The Study of Immunochemotherapy with PSK for Gastric Cancer; 95% CI: 95% Confidence Interval.

its trophic activity. Intramuscular vaccination with G17DT seems to prolong overall survival in certain patients affected by advanced gastric cancer [124].

Another way to utilize immunotherapy in gastric cancer patients as been analized by Kono et al. [125]. They reported that CTLs from gastric cancer patients, which are restricted to MHC class I, can react specifically against autologous tumor cells. Therefore, adoptive immunotherapy with tumor-associated lymphocytes (TALs) can have therapeutic activity and can induce regression of established tumors or micrometastases in adjuvant setting associated with chemotherapy. In advanced gastric cancer patients they reported an overall survival rate significantly better than in control group ($p < 0.05$), and
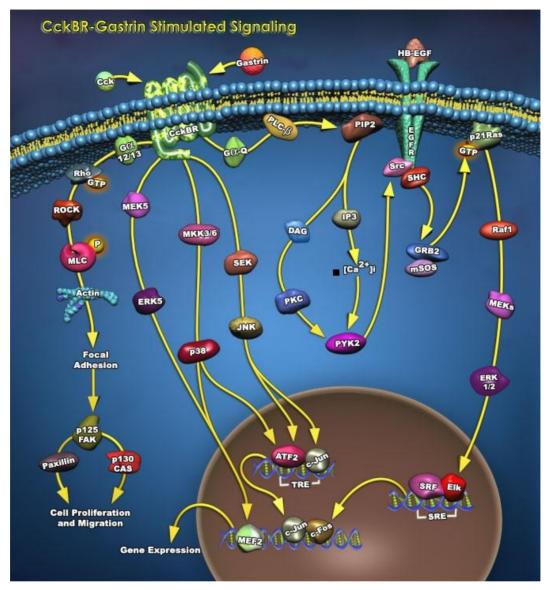
Figure 1. This chart illustrates gastrin pathway: signaling pathways associated with the gastrin-releasing peptide receptor (GRPR) in cancer. Gray lines indicate possible functional interactions. Black lines indicate established pathways activated upon GRPR stimulation.

adoptive immunotherapy is an indipendent prognostic factor for these patients, indicating that it influenced patient survival in a positive manner.

In a recent study Wu *et al.* [126] utilized Dendritic cells (DC) as a vector for immunotherapy in gastric cancer patients. DC are the professional antigen-presenting cells (APCs) specialized to initiate and regulate immune response. DC loaded with tumor antigens is able to induce protective antitumor responses and produce significant therapeutic immunity to established tumor. DC during their maturation develop the ability of antigen uptake and capture and of presenting antigen and release IL-12, that enhance both innate (NK cells) and acquired (B and T cells) immunity. The use of DCs pulsed with tumor

peptide or loaded with tumor cells lysate create vaccines which works against tumor and has been used in advanced gastric cancer patients. Gastric cancer seems to overexpress HER-2/neu and vaccination with DCs pulsed with HER-2/neu peptide intradermally results in tumor regression and decrease of tumor markers (CEA or Ca19-9).

More than 15 years ago, gene therapy emerged as a new therapeutic approach and has meanwhile become an important strategy in cancer treatment. The original concept of cancer gene therapy was further developed into two branches as a result of different strategies of therapeutic benefit: molecular cancer therapy and virotherapy. Molecular cancer therapy can be defined as a therapeutic

technique, which aims at the introduction of nucleic acids into cancer patients' cells in order to modulate the gene expression profile of the target cells and thereby eradicate the tumor. In contrast, virotherapy is a new concept of gene therapy that uses replication-competent oncolytic viral vectors (OVV) with viro-oncolytic potency for targeted tumor cells destruction. There are different ways to modulate tumor growth by gene therapeutic strategies. These include direct destruction of tumor cells, inhibition of tumor angiogenesis and tumor cell spread and activation of the host immune response against the tumor. The benefit of using cytokines can be improved if combined with other gene therapeutic options. For examples, synergistic anti-tumor effect was achieved by simultaneous expression of IL-2 and IFN-β, or by combining an oncolytic adenovirus (oAdV) with IL-12 immunotherapy. Zhang *et al.* evaluated the anti-tumor effects of the HSV-tk/GCV system (a suicide gene system: herpes simplex virus thymidilate kinase ganciclovir) together with the expression of recombinant IL-2 or TNF-α in gastric cancer [127]. Using similar protocols others groups found strongly enhanced anti-tumor effects after coexpression of IL-2, GM-CSF and HSV-tk/GCV in a gastric cancer model [128]. These results strongly indicate the potential impact of combined cytotoxic and immunomodulatory gene therapy in gastric cancer patients.

Other attempts were made in enhancing host's immune system against gastric cancer. We recently published the definitive results, with the full accrual of patients and a median follow-up of 51 months, of a randomized trial in which we evaluated the clinical benefit of patients treated with Interleukin-2 before undergoing surgery. There are data suggesting a biological and clinical benefit of subcutaneous, preoperative administration of low-dose IL-2 in colon cancer [129-131], which encouraged us to evaluate low-dose IL-2 therapy in the neoadjuvant setting for patients with gastric adenocarcinoma who underwent surgery and to evaluate its effects on systemic and tumor infiltrating lymphocyte numbers. We also sought to determine if neoadjuvant low-dose IL-2 could influence the clinical outcome for patients undergoing gastric resection for cancer.

IL-2 was first described as a T cell growth factor [132,133]. It is produced by activated T cells and stimulates the growth and function of T cells and NK cells. A decreased endogenous IL-2 production is known to occur in cancer patients with advanced stages, possible due to tumor-released factors such as IL-10, TGF-β and sIL-2r, and inducing a cell-mediated immunodeficiency status, which is demonstrated to be clinically relevant. In fact, either the total and the T-cell lymphocytopenia represents a prognostic factor for a poor survival in cancer patients, independent by other well known clinical prognostic factors such as performance status, disease extent, and

weight loss. Moreover, although radical surgery represents the only curative treatment, the biological response to major surgical trauma induces a temporary immunosuppression, likely due to the release of polipeptidic mediators having immunosuppressive activity and to the direct inhibition of circulating lymphocytes by monocytes/macrophages induced by laparotomic surgical stress. The postoperative period likely represents a critical time for the subsequent evolution of the disease, during which an effective cell-mediated immune response may eliminate the minimal neoplastic residual after macroscopic radically surgery; on the contrary an impaired immune surveillance can allow the growth of the few residual cancer cells, spread before and/or during surgery. In fact, in colorectal cancer patients, the lack of recovery of circulating T-cells in the late postoperative period is associated with a worse prognosis. The active managing of the immune response in the postoperative period may represent a powerful strategy for improving the therapeutic effectiveness of surgery: for this purpose, the administration of interleukin-2 the T-cell growth factor seems actually the most active cytokine, able to revert the cell-mediated immunodeficiency.

IL-2 was first used in patients with advanced cancer, and some clinical responses were seen [134], allowing FDA to licence IL-2 as a therapy in metastatic melanoma and metastatic renal cell carcinoma. However, extremely high doses were given in the initial cancer trials (up to 150 million units per day) resulting in severe toxicity, including capillary leak syndrome, hypotension and pulmonary edema. Many of the first patients treated on this high dose regimen required hospitalization in an intensive care unit. Because of its central role in T cell immunity, IL-2 was also one of the first immune-based therapies to be tested in patients with HIV. In these studies an intermediate dose was given. Kovacs *et al.* [135] administered 18 million units per day for 5 days every 8 weeks by continuous intravenous infusion in the hospital. The results were significant in patients with baseline CD4 counts >200 cells/mm^3. In addition, the infusion of IL-2 was still associated with side effects such as fever and flu-like symptoms. Subsequent trials of intermittent intermediate dose IL-2 have mainly used subcutaneous administration, since this seems to have similar efficacy with less severe side effects and allows outpatient administration [136]. In addition, some have argued for low dose, continuous IL-2 rather than intermittent IL-2 at higher doses [137]. In previous randomized studies on patients with advanced colorectal cancer undergoing surgery, the preoperative administration of IL-2 sc. for 3 days at a dose of 18 million UI/daily was active in the prevention of postoperative immunodepression and was effective in improving the overall survival in IL-2 treated patients vs control patients, with moderate toxicity.

More recently, studies on human immunodeficiency virus (HIV) patients, demonstrated that IL-2 at 9 million UI/daily, administered subcutaneously for 5 days every 2 months, induce significant activation and growth of T helper lymphocytes in patients with HIV related immunodeficiency. Subcutaneous administration is less toxic and warrants a better distribution, in lymphatic compartment, where the interaction between IL-2 and cell receptors occurs, in comparison with the intravenous administration route. Preoperative IL-2 administration at 18 million IU/daily subcutaneously for 3 days induced a significant durable lymphocytes increase and improved the survival in a series of colorectal patients in previous study, but such a treatment had to be performed as inpatient therapy so increasing the treatment costs. Lower IL-2 dosing administered in a shorter period might make this treatment feasible as outpatient, and less expensive. Moreover it has been postulated on clinical grounds that the anticancer effects of IL-2 are mediated through *in vivo* expression and activation of cytotoxic lymphocytes and/or promotion of their migration within target tissues. It is well known that most solid human neoplasms are infiltrated by poly- and mononuclear cell infiltrate [138, 139]. This tumor infiltrating lymphocytes (TILs) has been recognized as a tumor-host reaction in various neoplasms [140,141] and usually contains a predominantly T lymphocytic infiltrate associated with the host's specific immune response to the neoplasm. The degree of infiltration has been suggested to be a measure of the host immune response. An increased TIL, with high number of activated cytotoxic lymphocyte, have been linked to prognosis in some tumors like colorectal carcinoma, medullary carcinoma of the breast and malignant melanoma with a clear positive correlation between the density of the lymphoid infiltrate at the advancing margin of the tumor and the prognosis of the patients.

3. Discussion

Our personal experience in gastric cancer immunotherapy.

In recent years many trials were conducted with different IL-2 regimens in solid tumors other than melanoma and renal cell carcinoma with variable results [142]. IL-2 was also used to improve the anti-tumor activity of other forms of immunotherapy [143-150] chemotherapy [151-156] and surgery [157-163].

Immunotherapy with IL-2 may thus also be a potentially promising alternative strategy for gastric cancer. We enrolled 68 patients with assessable gastric cancer, who met eligibility criteria for IL-2 administration and surgical procedures, between October 1999 and July 2003 [164]. Patients were randomized in two groups: one was treated before surgery with low doses IL-2 (cases), the other underwent surgery without having been treated

(controls). Low dose IL-2 was administered subcutaneously at 9,000,000 UI twice a day for three days. Patients underwent gastric surgery 36 hours after the last dose. Other than characterize the peripheral blood count, such as Total peripheral WBC, neutrophils, total lymphocytes (CD3+), T helper lymphocytes (CD4+), cytotoxic lymphocytes (CD8+) and Natural Killers (NKs), we also looked at the peritumoral infiltration and we graded its density according to the classification of Ropponen modified [165] and four grades were established, for both lymphocytic, eosinophylsic, and neutrophilic infiltration: negative (0: 0 cells/field), weak (+: 1 - 30 cells/field), moderate (++: 30 - 50 cells/field), dense (+++: >50 cells/field).
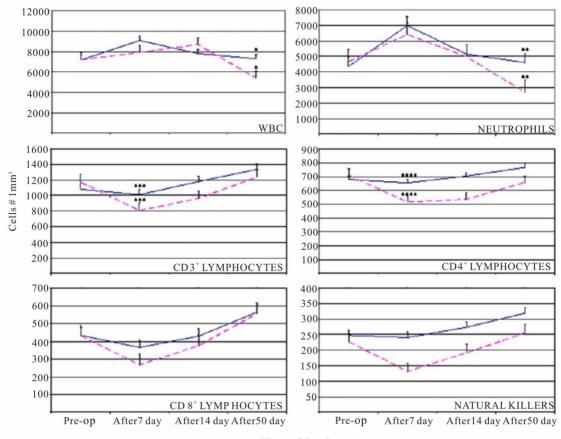
Patients' age, sex, site of tumor, type and grading of tumor, stage of tumor, type of surgical intervention and total months of follow up, were not significantly different in the two groups. Thirty patients (44.1% of the total), fifteen cases and fifteen controls, underwent partial gastrectomy; thirty-eight (55.9%), twenty-one cases and seventeen controls, underwent total gastrectomy. The surgical choice was determined by tumor site. Total gastrectomy was preferred to partial gastrectomy when the tumor was localized to the cardia.

Pre-operative administration of low-dose IL-2 resulted in very mild toxicity, which mostly consists in fever and common flu-like symptoms, skin erythema at the injection site which spontaneously disappeared in a few days.

Our data demonstrated a decrease in total peripheral blood lymphocytes below 1000 cells/mm^3 (805 ± 446 lymphocytes/mm^3) 7 days after surgery starting from a mean baseline value of 1172 ± 428l ymphocytes/mm^3 for those patients who were not pre-treated with IL-2. We also observed a specific decrease in CD4+ T, CD8+ T and NK cell counts after operation (**Figure 2**). This decrease after tumor manipulation during surgery might provide a fertile environment for residual tumor cells to grow or for micrometastasis to develop. It is not known if improvements in surgical technique and keeping tumor manipulation to a minimum during operation will prevent the decrease in lymphocyte depletion post-operatively. Seven days after operation we noted a minor decrease in peripheral blood CD3+ T cells, CD4+ T cells, CD8+ T cells and NK cells in patients pre-treated with low-dose IL-2 compared to control patients (**Figure 2**). This minor decrease was statistically significant for CD3+ and CD4+ T cells ($p < 0.05$). While we also observed a trend toward decreasing in CD8+ T cells and NKs in control patients, this did not reach statistical significance due to the high variability within the population. After 7 days, lymphocyte counts in IL-2-treated and control patients increased at a similar rate suggesting that the activity of IL-2 is most pronounced in the first week after surgery. In general, surgical trauma induces lymphopenia with a slow
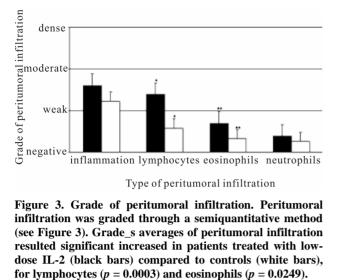
Figure 2. Total WBC, neutrophils, CD3+ T, CD4+ T, CD8+ T and NK cells counts were assessed by flow cytometry on peripheral blood samples preoperatively (Pre-op) and on days 7, 14 and 50 after surgery. A significantly higher number ($p < 0.05$) of total WBC and neutrophils was noted in cases (continuous line) compared to controls (dashed line) on 50th day after surgery. Seven days after surgery, the decrease in CD3+ and CD4+ T cell number was significantly lower ($p < 0.05$) in cases than in controls.

rebound depending on the type and severity of trauma. IL-2 appears to set a higher starting point for the lymphocyte rebound after trauma. In addition to peripheral blood lymphocytes, we also analyzed the local peritumoral cellular infiltrates.

Our data suggested that there was an increase in lymphocytic and eosinophilic infiltration in patients pre-treated with IL-2 compared to controls (see **Figure 3**). These data imply that IL-2 might act to abolish the post-operative decrease in peripheral blood lymphocytes and also increase the local accumulation of lymphocytes and eosinophils within the tumor. This is consistent with data from melanoma patients where IL-2 has been shown to induce lymphocytic infiltration into tumors of patients who achieved clinical responses [166]. An increased in eosinophilic infiltrates have also been reported in patients with colon cancer pre-treated with IL-2 [167]. The activity of IL-2 on peritumoral lymphocytes and eosinophils is perhaps not unexpected and may result from direct action on the IL-2 receptor, which is expressed by both

activated lymphocytes and eosinophils [168], and an indirect action due to an IL-2-mediated cascade of cytokines and chemokines that results in lymphocyte and eosinophil migration and proliferation. The presence of peritumoral infiltrating lymphocyte is a well described phenomenon in cancer patients and typically relates to a better prognosis as reported in patients with primary malignancies of the lung [169], liver [170], ovary [171], colon [172,173], genitourinary tract [174] and stomach [175]. IL-2 seems not to modify the composition of lymphocytic infiltration, which resulted to be mostly made by T cells, widespread diffused into the tumor, and by B cells, peripherally organized in a nodular shape. Although the changes in lymphocyte frequency with neoadjuvant IL-2 were interesting, we did not notice an improvement in relapse-free or overall survival. Stepwise multivariate analyses were made to evaluate factors that influenced survival and found that tumor stage and patient age were the only predictors of response in our patients. It was not unexpected that a better prognosis was

Figure 3. Grade of peritumoral infiltration. Peritumoral infiltration was graded through a semiquantitative method (see Figure 3). Grade_s averages of peritumoral infiltration resulted significant increased in patients treated with low-dose IL-2 (black bars) compared to controls (white bars), for lymphocytes ($p = 0.0003$) and eosinophils ($p = 0.0249$).

related with a low tumor stage and younger age at presentation. Although less significant, overall survival also appeared to be affected by the type of surgical intervention. Total gastrectomy resulted in a worse prognosis than partial gastrectomy and this might relate to the fact that total gastrectomy was chosen more frequently for cardia tumors, which have been described as being more aggressive than tumors affecting other parts of the stomach. In addition these results might also relate to the fact that gastrectomy is a bigger procedure and may lead to more profound immunosuppression, although we did not find this in our study. The fact that relapse-free survival did not depend on the type of surgical intervention might have been related to appropriate selection of patients for surgical intervention and good operative technique.

4. Conclusion

According to our data low doses of IL-2 administered pre-operatively to patients with gastric cancer activate peripheral and peri-tumoral lymphocytes but did not affect prognosis and Kaplan-Meier analysis suggested no differences between cases and controls (**Figure 4**). The reasons for the lack of therapeutic effectiveness are not entirely clear but we would propose several points for consideration. Firstly, we know that only selected melanoma and renal cell carcinoma patients have a significant benefit from high-dose IL-2 therapy and for this reason we may require a much larger study to detect a benefit in patients with gastric carcinoma. Secondly, we utilized a low-dose IL-2 regimen and most studies with IL-2 have found that high-dose therapy is superior to low-dose treatment regimens [176]. Finally, the influence of IL-2 on regulatory T cells may be critical for influencing clinical outcome and we did not directly examine these cells in the current study. While many reports suggest that these regulatory T cells are elevated in cancer pa-

Figure 4. *Kaplan-Meier* curves shows that there is no significant difference in overall survival and in relapse-free survival, between patients treated with low-dose IL-2 (*dark line*) and controls (*light line*).

tients, there are some data suggesting that high-dose IL-2 may be associated with a decrease to normal levels of this population after exposure to high-dose IL-2 [84]. Thus, further investigation with high-dose IL-2 and careful monitoring of regulatory T cell populations may provide further insights into the utility of IL-2 and other forms of immunotherapy for patients with gastric cancer.
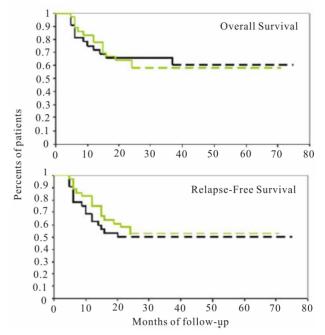
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