THE EFFECTS OF NON-STEROIDAL ANTIINFLAMMATORY DRUGS (NSAIDS) ON OESOPHAGEAL CANCER

A thesis submitted for degree of Doctor of Philosophy

By

Liu Jun Feng

September 2006

Content

List of Tables

List of Figures

Abstract

The aim of this study was to investigate COX-2 expression in squamous cell carcinoma of the oesophagus (SCC), and the potential of non-steroidal anti-inflammatory drugs, which inhibit the action of the enzyme, for chemoprevention of this cancer. The epidemiology of SCC and the outcome from surgery for this disease in Hebei Province, China, were reviewed. The rate of postoperative complications and deaths following oesophagectomy fell steadily over the last five decades, but the long-term survival remained disappointing. Improved survival is likely to be dependent on earlier diagnosis and better adjunctive therapies.

Tissue was obtained from patients who had an oesophagectomy for SCC over 20 years earlier. The expression of COX-2 was elevated and correlated with TNM stage and lymph node metastases. Survival was longer in those patients whose tumours expressed lower levels of COX-2.

The mechanism of action of aspirin, a non-selective COX inhibitor, and NS-398, a selective COX-2 inhibitor, was investigated *in vitro*. Both drugs inhibited the proliferation of and induced apoptosis in the SCC cell line TE-13. These changes correlated with a reduction in COX-2 mRNA and protein expression, prostaglandin synthesis, inhibition of NF-KappaB nuclear translocation and an increase in cytoplasmic IKappaB. Similar changes were seen in tumour tissue resected from patients given the selective COX-2 inhibitor Mobic daily for 14 days before surgery.

These results suggested that aspirin and similar drugs might have value in cancer therapy. A clinical trial was established to determine if treatment with aspirin post-operatively would improve survival of patients who had had an oesophagectomy for SCC. Preliminary results suggested that treatment had no effect on survival in patients operated on for SCC.

Publications related to this thesis

1. Liu JF, Wang QZ, Hou J. Surgical treatment for cancer of the oesophagus and gastric cardia in Hebei, China. Br J Surg 2004;91:90-98.
2. Liu JF, Jamieson GG, Drew PA, Zhu GJ, Zhang SW, Zhu TN, Shan BE, Wang QZ. Asprin induces apoptosis in oesophageal cancer cells by inhibiting the pathway of NF-kappaB downstream regulation of cyclooxygenase-2. ANZ J Surg 2005;75: 1011 – 1016.
3. Liu JF, Jamieson GG, Wu TC, Zhang SW, Wang QZ, Drew PA. COX-2 expression in SCC of the oesophagus. Diseases of the esophagus 2006;19(5):314-319.

Signed Statement

This work contains no material which has been accepted for the award of any other degree ordiploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

I give consent to this copy of my thesis, when deposited in the University Library, being available for loan and photocopying.

SIGNED: DATE: 11-05-2007

Acknowledgements

I would like to express my sincere gratitude to my supervisors Professor Glyn G Jamieson and Dr. Paul Drew, for sharing their knowledge and expressing encouragement during the course of this project. I am also grateful for their comment and advise during the preparation of this thesis. Thanks specifically due to Professor Glyn G Jamieson for kindly providing tuition to me, without his funding I could not have completed my PhD term. Furthermore, I would like to thank Dr. David Watson, Mr. Eric Smith and Mrs. Christine Bates-Brownsword for their help during my stay in Adelaide.

I would further express my gratitude to Professor Shan BE and Zhu TN at the Scientific Research Centre of Hebei Medical University for their kind support during I was conducting cell biological study. I would also like to thank Professor Zuo LF for his meticulous instruction when performing flow cytometry. Also, I would like to thank Professor Yu FL, Professor Wang XL and Mrs Yang HC for their help in performing immunohistochemistry and grading the extent of staining for COX-2. I also take the opportunity to thank Professor Gao XS for providing cell line of squamous cell carcinoma of the oesophagus, TE13 and others.

I would extend my gratitude to my dear colleagues Professor Wang QZ, Professor Li BQ, Professor Wang FS, Dr. Cao FM, Dr. Tian ZQ and Dr. Zhang YF for their assistance while I was conducting the clinical trial of aspirin on oesophageal cancer. Many thanks to Dr. Zhu GJ, Dr. Zhang SW and Dr. Wu TC for their assistance with performing cell biological tests.

At last, I would like to thank my wife, Dr. Li SB and my parents for their unconditional love and support.

Chapter 1. Literature review

1.1. Epidemiological differences in oesophageal cancer between China and the West

Oesophageal cancer is the seventh most common malignancy worldwide (Parkin et al., 1999). It has a poor prognosis, with a high morbidity and mortality. In China it is the fourth most frequent cancer (Li et al., 1996), while in Hebei Province in China, the disease is the most common cancer (Hou et al, 1995). In the southern area of Hebei province the incidence of carcinoma of the oesophagus is reported to be the highest in the world at 199 cases per 100,000 population (Hou et al., 1998), over 95% of which are squamous cell carcinoma (Li and Yao, 1997).

Is oesophageal cancer the same disease in the West and the East? The most striking observation over the past decades is its changing epidemiological pattern. In the West, adenocarcinomas of the oesophagus and gastric cardia are increasing at an alarming rate. The reason for this rapid increase in incidence is uncertain, but is widely believed to be related to an increase in gastroesophageal reflux disease (GERD) and Barrett's oesophagus (Blot et al., 1991; Blot et al., 1993; Pera et al., 1993; Pera et al., 2005; DeMeester, 2006). In the East, oesophageal cancers are predominantly squamous cell carcinoma (SCC), and mostly located in the middle third of the oesophagus, in contrast to the lower third for adenocarcinomas.

There is also a great variation in the occurrence of SCC itself in different parts of the world. The mortality rates for SCC in Japan, Europe and the USA are relatively low, typically ranging from 2-15 per 100,000 (Miller, et al., 1992; Muir et al., 1987). Areas of high incidence for SCC include Northern China (Li et al., 1980; Li et al., 1982), Northern Iran (Kmet and Mahboubi, 1972; Mahboubi et al., 1973), the Central Asian Republics (Zaridze et al., 1992), and South Africa (Jaskiewicz et al., 1987).

However, within China, the distribution of SCC varies from region to region. The areas of highest incidence are predominantly located in North China's Taihang Mountain areas (Shao, 2002). Even within Hebei Province the incidence of SCC varies between different regions. From the south to the north of Hebei the incidence decreases gradually. The area with the highest incidence is in the southernmost Cixian county, 125.34/100,000 for males and 69.25/100,000 for females, while the region with the lowest incidence is in Chicheng County, 5.1/100,000 for males, and 1.4/100,000 for females (Ma et al., 1989).

The aetiology of SCC appears to differ between the high- and low-risk areas. Alcohol and tobacco use are thought to be the major risk factors in Western societies, with diet playing an additional, but less prominent role (Pottern et al., 1981; Ziegler et al., 1981; Tuyns, 1982). Diet and other factors appears to be more important in the high-risk areas such as China (Li et al., 1980; Yang, 1980; van Rensburg, 1981; van Rensburg, 1987; Anonymous, 1997; Li et al., 2001), although the precise risk factors in these population remain elusive. The high-risk areas are generally rural and poor, with dry climates and infertile soils. Diets are typically monotonous, with corn or wheat as the staple foodstuff (van Rensburg, 1981; van Rensburg, 1987). There is usually little consumption of fruits, vegetables and animal foods, leading to widespread inadequacies of vitamins and minerals, including riboflavin, vitamin A, vitamin C, zinc, and others (Yang et al., 1984). In addition to dietary deficiencies, some dietary exposures in high-risk areas may be involved in causing oesophageal cancer, while others may predispose to neoplasia by physically damaging the oesophageal mucosa. Potentially carcinogenic dietary exposures include consumption of picked vegetables and fungus-contaminated corn in China (Li et al., 1980; Yang, 1980; Lu et al., 1981; Li, 1982; Luo et al., 1990; Cheng et al., 1992; Wang et al., 1992). Dietary factors which may damage the oesophageal mucosa include ingestion of millet which contains silica fragments (O'Neill et al., 1986) and consumption of scalding hot liquids (Yang, 1980; Ghadirian, 1987; Cheng et al., 1992). In addition to these environmental factors, genetic predisposition may be

important in some high-risk areas.

1.2. The results from surgical treatment of oesophageal cancer in China and the West

It is noteworthy that the postoperative mortality and morbidity rates in the East are, in general, lower than in Western countries. The reason for this is not immediately apparent. With the change in the epidemiology of oesophageal cancer, the cases seen may be different between the East and the West. In the West the incidence of adenocarcinoma is rising, comprising over 80% of oesophageal cancer in some clinics (Visbal et al., 2001). In the East, over 96% of oesophageal cancers are SCC (Li and Yao, 1997). A study in Germany compared patients with SCC to those with adenocarcinoma in their preoperative risk stratification. More patients with SCC were 'blue collar' workers, while those with adenocarcinomas tended to be 'white collar' workers. Alcohol and tobacco consumption were higher in patients with SCC, and they were more likely to have compromised pulmonary or hepatic function. In contrast, patients with adenocarcinomas were more likely to be overweight, with a higher body mass index, and have impaired cardiac function (Bollschweiler et al., 2000). It is unclear whether patients in Asia, who have predominantly squamous cancers, are different in risk analysis when compared to patients with adenocarcinomas in the West. However, regardless of geography, a low postoperative mortality rate of less than 5% can be obtained in selected centers (DeMeester et al., 1988; Akiyama et al., 1994; Zhang et al., 1994; Orringer et al., 1999; Whooley et al., 2001). Patient selection and better perioperative care may have contributed to improvement in surgical results (Watson and Allen, 1994; Tsui et al., 1997). There is also no doubt that a significant learning curve exists for the surgeon doing the oesophagectomy, and the results are proportional to the surgical experience attained. Anastomotic leakage, one of the most lethal complications of oesophagectomy, can be shown to decrease substantially with increase in surgical experience. In the Department of Thoracic Surgery, Fourth Hospital, Hebei

Medical University, postoperative mortality dropped from 4.6% to 1.1% between 1952 and 2000, along with a reduction in the anastomotic leakage rate from 5% to 3.4% (Liu et al., 2004).

There are 2 aspects of the surgical treatment for oesophageal cancer which remain contentious, one is appropriate approach for resection of the oesophagus (Law, 2001) and the other is the extent of lymphadenectomy required (Law, 1999). Obviously, the surgical approach will have an impact on the extent of lymphadenectomy possible. Some surgeons perform a limited resection for oesophageal cancer, believing that the surgery is usually palliative and only provides a chance of cure for early disease (Orringer et al., 1999). On the other hand, some surgeons perform extended cancer resection and lymphadenectomy, even for patients without apparent metastasis to regional lymph nodes (Akiyama et al., 1994).

As mentioned, the incidence of adenocarcinoma in the lower third of the oesophagus has been increasing in the West. For this form of oesophageal cancer a transhiatal oesophagectomy with wide opening of oesophageal hiatus, and radical lymphadenectomy in the upper abdomen and lower mediastinum, are usually performed (Alderson et al., 1994; Pinotti et al., 1997). In the East, especially in China, over 70% of oesophageal cancers are located in the middle third of the oesophagus, thus a thoractomy is usually performed in these patients (Li and Yao, 1997). A three-field lymphadenectomy including the lymph nodes in the neck, the mediastinum and the upper abdomen could be considered reasonable, because a high incidence of lymph node metastases in the neck has been found in this proximal oesophageal cancer (Aciyama et al., 1994). However, this technique is rarely used, except in Japan, for both retrospective and randomized studies have not demonstrated that it provides any survival benefits (Adachi et al., 1996; Nishihira et al., 1998). Unfortunately, three-field lymphadenectomy has resulted in higher postoperative mortality and morbidity in some clinical studies (Siewert and Stein, 1999). In a review which summarized 44 series published between 1986 and 1996, covering

2675 patients undergoing transhiatal oesophagectomy and 2808 patients transthoracic oesophagectomy, no significant differences were found in postoperative morbidity, mortality, or long-term survival, between the 2 groups (Rindani et al., 1999).

Over the past several decades studies on adjuvant and neo-adjuvant therapy for oesophageal cancer have been conducted worldwide to improve local control and long-term survival. Up to now, however, randomized phase III trials of neo-adjuvant radiotherapy have failed to show an increased rate of resection or improved long-term survival compared to surgery alone (Launois et al., 1981; Gignoux et al., 1987; Wang et al., 1989; Arnott et al., 1992; Nygaard et al., 1992; Fok et al., 1994; Rindani et al., 1999; Greer et al., 2005). Although chemotherapy is well tolerated by the majority of patients, there is no evidence for increased survival from neo-adjuvant chemotherapy (Adachi et al., 1996; Bhansali et al., 1996; Urschel et al., 2002). For this reason, neo-adjuvant chemoradiotherapy has been intensively studied in recent years (Engel and Holzel, 2001; Fietkau and Kundt, 2001; Slater et al., 2001; Imdahl et al., 2002). While this modality results in a complete pathological response in 24-42 % of patients (Geh et al., 2001), and a downstaging of the oesophageal cancer (Slater et al., 2001), consensus on its survival benefit has not been reached (Slater et al., 2001).

1.3. Non-steroidal anti-inflammatory drugs in the prevention of cancers

1.3.1. Evidence for preventive effects of NSAIDs in cancer

The discovery of new chemotherapeutic agents and improvements in radio-chemotherapeutic regimens may increase survival for cancer patients. Salicylic acid has been extensively used for relief from pain and fever (Elwood, 2001), and its preventative effects on some cardiovascular and cerebrovascular diseases have been known for several decades (Manson et al., 1991; Goodnight, 1996; Elwood et al., 1998). In recent years the use of aspirin and other NSAIDs has been

reported to reduce the occurrence of a variety of cancers, including oesophageal cancer (Farrow et al., 1998). Many studies have reported that NSAIDs exert their anti-cancer effects via the inhibition of the cyclooxygenase (COX) enzymes, COX-1 and/or COX-2 (Alberts et al., 1995; Lupulescu, 1996; Attiga et al., 2000).

A number of non-randomised studies have reported that people who regularly use aspirin or other NSAIDs have a lower incidence of adenomatous polyps and lower incidences of or deaths from colorectal cancer compared with non-users (Deutsch, 1992; Gaut, 1993). Sustained use of NSAIDs has been reported to be associated with a 30-50% reduction in adenomatous polyps, incident disease and death from colorectal cancer (Paganini-Hill et al., 1991; Paganini-Hill, 1995; Kauppi et al., 1996; Sandler et al., 1998). Retrospective studies have demonstrated a 40-50% risk reduction of colorectal cancers in NSAID users (Kune et al., 1988; Rosenberg et al., 1991; Suh et al., 1993; Peleg et al., 1994; Reeves et al., 1996). Prospective studies have also shown a reduction in the incidence of and mortality from colorectal cancer in subjects who have used these compounds (Kune et al., 1988; Rosenberg et al., 1991; Thun et al., 1991; Gann et al., 1993; Suh et al., 1993; Giovannucci et al., 1994; Peleg et al., 1994; Schreinemachers and Everson, 1994; Kauppi et al., 1996; Reeves et al., 1996; Rosenberg et al., 1998; Sturmer et al., 1998). The results of epidemiological studies have suggested that the duration and continuity of NSAID use may be more critical than the daily dose (Thun and Heath, 1995; Collet et al., 1999; Smalley et al., 1999). NSAIDs have also been reported to reduce the risk of cancers of the oesophagus (Farrow et al., 1998)， stomach (Thun et al., 1993), breast (Thun et al., 1993; Schreinemachers and Everson, 1994; Egan et al., 1996; Rosenberg, 1996), lung (Schreinemachers and Everson, 1994), prostate (Bucher et al., 1999), urinary bladder (Thun et al., 1993) and ovary (Thun et al., 1993; Cramer et al., 1998).

In some studies in rodents, aspirin, and other conventional NSAIDs such as piroxican, indomethecin, sulindac, ibuprofen, and ketoprofen and selective COX-2 inhibitors such as celecoxib, have inhibited chemically induced carcinogenesis (Craven and

DeRubertis, 1992; Reddy et al., 1993; Barnes et al., 1997; Kawamori et al., 1998; Li et al., 1999). Taketo (1998a) crossed heterozygous APC (delta716) knockout mice (a mouse model of human familial adenomatous polyposis) to COX-2 gene knockout mice. The double knockout mice for both APC (delta716) and COX-2 genes had marked reduction in the size and frequency of intestinal polyps, as did the APC (delta716) knockouts fed a selective COX-2 inhibitor (Oshima et al., 1996). Thus blocking the action of COX-2, either by the introduction of a COX-2 gene mutation, or feeding with the COX-2 selective inhibitor to the APC (delta716) knockout mice, reduced the number and size of intestinal polyps dramatically.

Increased levels of COX-2 have been reported in carcinomas of the colon (Sano et al., 1995; Fujita et al., 1998), as well as stomach, breast, oesophagus, lung, liver and pancreas (Ristimaki et al., 1997; Hida et al., 1998; Hwang et al., 1998; Wolff et al., 1998; Koga et al., 1999; Okami et al., 1999; Tucker et al., 1999; Zimmermann et al., 1999). In contrast, most studies report that the levels of COX-1 are similar between normal and tumour tissues (Sano et al., 1995). These findings suggest that COX-2 may be associated with carcinogenesis and/or progression of certain types of human malignancies.

1.3.2. Anticancer mechanisms of NSAIDs

1.3.2.1. COX-2 and tumourigenesis

In the 1970s Vane and associates found that cyclooxygenase (COX) had 2 isoforms, COX-1 and COX-2 (Vane, 1971). COX-1 is expressed constitutively within many normal tissues and is thought to be responsible for the maintenance of normal physiological function, such as cytoprotection of the stomach, vasodilation in the kidney (Vane, 2000), and control of platelet aggregation. In contrast, COX-2 is not expressed normally, but is rapidly induced in response to proinflammatory and mitogenic stimuli including cytokines, endotoxins, interleukins and phorbolester (DuBois et al., 1994; Hempel et al., 1994; Prescott and White, 1996), and thus is

thought to be responsible for pathological changes. Many more recent studies have further highlighted the relevance of COX-2 to human carcinogenesis.

COX-1 and COX-2 are rate-limiting enzymes for the formation of prostaglandins. The substrate for both COXs, as well as the lipooxygenase (LOX) enzymes, is arachidonic acid, an essential polyunsaturated fatty acid consumed in the diet or derived from elongation and desaturation of dietary linoleic acid (Marnett, 1992). Arachidonic acid can also be formed from the hydrolysis of phospholipid precursors catalysed by the enzyme phospholipase A_2. The COX enzymes introduce 2 molecules of O_2 into the arachidonic acid to form prostaglandin (PG) endoperoxides, from which PGH_2 is formed. PGH_2 is an intermediate product that may be converted to biologically active prostaglandins (PGE_2, PGD_2, PGF_2, PGI_2, etc.) and thromboxanes (e.g., TxA_2) by various specific synthases or reductases. PGH_2 may also be converted to malondialdehyde (MAD) and 12-hydroxyhepetadec (HHT), spontaneously or via catalysis by thromboxyane synthases or specific cytochrome p450s (Sharma, 2002). MAD, a by-product of prostaglandin biosynthesis, is a mutagen and carcinogen (Sharma, 2002). Under physiological conditions MAD reacts with DNA to form adducts, predominantly with deoxyguanosine to generate pyrimidopurinone-deoxyguabosine adducts (M1G) (Marnett et al., 1986). M1G, thought to be mutagenic, has been detected in a variety of human tissue at a concentration of 3-150 adducts/10^8 nucleotides (Marnett, 1999).

COX-2 has been reported to contribute to tumourigenesis and the malignant phenotype of tumour cells through one or more of the following several mechanisms: (i) increasing the production of prostaglandins, (ii) converting procarcinogens to carcinogens, (iii) inhibiting apoptosis, (iv) promoting angiogenesis, (v) increasing the invasiveness of cancer cells, or (vi) modulating inflammation and immunoresponsiveness (Dempke et al., 2001; Xu, 2002).

COX-2 derived PGE_2 is a pro-inflammatory agent, and is the major prostaglandin produced in many human solid tumours, including cancer of the colon (Rigas et al.,

1993), stomach (Uefuji et al., 2000), and breast (Rolland et al., 1980). There is much evidence that PGE_2 promotes tumour growth. Thus treatment with prostaglandin receptor agonists reversed NSAID induced adenoma regression in Apc (Min/+) mice (Hansen-Petrik et al., 2002), and PGE_2 significantly enhanced carcinogen induced colonic tumour incidence and multiplicity in rats (Kawamori et al., 2003) and increased intestinal adenoma burden in Apc (Min/+) mice (Wang et al., 2004).

PGE_2 has been shown to increase synthesis by macrophages of vascular endothelial growth factor (VEGF), which promotes tumour vascularisation. The increase in VEGF production can be mediated by both specific PGE receptor and PPAR-gamma mediated mechanisms. PGE_2 can transactivate epidermal growth factor receptor (EGFR), which results in stimulation of cell migration through increased PI3K-Akt signaling in colorectal cancer cells (Sheng et al., 2001; Pai et al., 2002; Buchanan et al., 2003). PGE_2 can inhibit apoptosis by inducing the expression of anti-apoptotic proteins such as BCL2 (Sheng et al., 1998), and increasing NF-KappaB transcriptional activity (Poligone and Baldwin, 2001), which is a key regulator of antiapoptotic pathways. PGE_2 also can transactivate peroxisome proliferator-activated receptor-delta (PPARD) which in turn promotes tumour cell survival (Wang et al., 2004). Ras is an oncogene and its activation is found in a wide variety of human malignancies. The Ras-MAP kinase cascade is one of the major intracellular signalling pathways responsible for cell proliferation. PGE_2 has been found to activate a Ras-MAPK pathway which in turn upregulates COX-2 expression in a self-amplifying loop, and stimulates colorectal cancer cell proliferation (Wang et al., 2005). PGE_2 also can downregulate Th1 cytokines (tumour necrosis factor α, interferon γ, and interleukin (IL)-2) (Harris et al., 2002) and upregulate Th2 cytokines such as IL-4, IL-10, and IL-6 (Della et al., 1997; Huang et al., 1998; Shreedhar et al., 1998). Moreover, PGE_2 can modulate immune function through inhibiting dendritic cell differentiation and T cell proliferation, and suppressing the antitumour activity of natural killer cells and macrophages (Goodwin

and Ceuppens, 1983; Yang et al., 2003). PGE_2 has also been demonstrated to upregulate complement regulatory protein decay accelerating factor (Holla et al., 2005). Thus the combined effects of PGE_2 on different components of the immune system may play a role in assisting neoplastic cells to evade immune attack.

1.3.2.2. Effects of NSAIDs on cancer

1.3.2.2.1 COX-2 dependent pathways

Because COX-2 has the potential to play a very important role in tumourigenesis and tumour progression in a variety of cancers, the ability of NSAIDs to inhibit the COX-2 could explain their anti-cancer effects. A summary of the reported effects of NSAIDs in cancer includes the inhibition of cellular proliferation and tumour growth, induction of apoptosis, reduction of angiogenesis, prevention of procarcinogen activation and augmentation of immune response (Lupulescu, 1996; Shiff et al., 1996).

Sheng demonstrated that treatment with COX-2-selective inhibitors induced apoptosis in colorectal cancer cells expressing COX-2 but not COX-1 (Sheng et al., 1998). Studies *in vitro* with non-selective NSAIDs including salicylic acid, sulindac, sulindac sulfide, aspirin, indomethacin, naproxen, and piroxicam indicated that they have pro-apoptotic properties (Shiff et al., 1995; Shiff et al., 1996; Elder et al., 1997; Sheng et al., 1998). The antiproliferative effects of NSAIDs are controversial, and the data is largely limited to *in vitro* studies. Shiff (1995) demonstrated the *in vitro* antiproliferative properties of NSAIDs, including sulindac and sulindac sulfide, on a human colon cancer cell line, HT-29. Subsequent studies using other nonselective NSAIDs such as aspirin, indomethacin, naproxen, and piroxicam, as well as selective COX-2 inhibitors, confirmed the antiproliferative effects of these drugs on colon cancer cell lines *in vitro* (Goldberg et al., 1996; Shiff et al., 1996; Qiao et al., 1997). However, Craven and DeRuberitis found an increase in proliferation in an induced colonic tumour in rats treated with aspirin (Craven and DeRubertis, 1992).

Solid tumours larger than 2 mm in diameter must stimulate the formation of new capillary blood vessels to support further growth by the mass (Masferrer et al., 1996; Holash et al., 1999; Jones et al., 1999). COX-2 expression is widely induced in the angiogenic vasculature of colorectal adenomatous polyps and in carcinomas of the colon, lung, breast, oesophagus, and prostate (Holash et al., 1999; Masferrer et al., 2000). PGE_2 stimulates angiogenesis, and recent studies have demonstrated that tumour growth is dependent on PGE_2 (Form and Auerbach, 1983; Hanahan and Folkman, 1996). Tsujii and colleagues demonstrated that cyclooxygenase affects colon carcinoma-induced angiogenesis by 2 mechanisms: COX-2 modulates the production of angiogenic factors, which stimulate endothelial tube formation, while COX-1 regulates angiogenesis in endothelial cells (Tsujii et al., 1998). Masferrer (2000) reported that COX-2, but not COX-1, derived prostaglandins regulate tumour-induced angiogenesis in mice implanted with human tumours. Celecoxib blocked the angiogenesis and suppressed tumour growth, consistent with the use of this anti-inflammatory drug in the treatment of human cancer (Masferrer et al., 2000). In model system in which tumour cells were implanted into mice, Williams noted that COX-2 in the host cells (i.e., the tumour stromal cells) appeared to be the more important influence on tumour growth. Therapeutic concentrations of COX inhibitors also suppressed the release of angiogenic growth factor by human or rodent colorectal cancer cells that were cocultured with vascular endothelial cells (Tsujii et al., 1998) and inhibited the growth of several human tumours transplanted into mice (Masferrer et al., 2000; Williams et al., 2000).

Reduced expression of HLA class I and II antigens has been reported in colonic tumours and in the adjacent normal mucosa (McDougall et al., 1990; Tsioulias et al., 1992; Tsioulias et al., 1993). PGE_2 can reduce the expression of these antigens, as well as suppressing T-cell proliferation, lymphokine production, macrophage activation, and T cell-mediated cytoxicity (Levy, 1997; Shiff and Rigas, 1997; Ahnen, 1998). These actions may assist the tumour to escape normal immune surveillance. By inhibiting prostaglandin synthesis, NSAIDs may up-regulate the expression of

major histocompatibility complex antigens, which has been demonstrated in animal models such as the azoxymethane-induced rat colonic tumour (Rigas et al., 1994), and by this and other effects may indirectly enhance the immune response to a tumour (Husain et al., 2002).

Cyclo-oxygenase enzymes may promote cancer by means other than the synthesis of prostaglandins. These enzymes can metabolize procarcinogens such as polycylic hydrocarbons, aflatoxins, halogenated pesticides, amines, and phenols, and convert them to active carcinogens (Levy, 1997). NSAIDs could protect against cancer by blocking this conversion of a procarcinogen to a carcinogen.

1.3.2.2.2 COX-2 independent pathways

In addition to COX-dependent pathways, some studies have shown that NSAIDs, including COX-2 inhibitors, may exert anti-tumour effects by pathways which are unrelated to the inhibition of COX activity (Grosch et al., 2001; Tegeder et al., 2001). The following two observations support the concept of COX-independent effects of NSAIDs: (i) the dose of NSAIDs used is usually much higher than that needed to inhibit COX-2 enzymatic activity and, (ii) NSAIDs are effective against cancer cells that do not express COX-2 (Marx, 2001). For example, both sulindac sulfide and piroxicam induced apoptosis in COX-2 expressing HT-29 human colon cancer cells as well as the COX-2 deficient HCT-15 human colon cancer cells. Treatment of HCT 15 cells with various prostaglandins did not reverse the apoptotic effects of the drugs in the HCT 15 cells, suggesting a COX-independent effect (Hanif et al., 1996).

NSAIDs can antagonise the anti-apoptotic activity of peroxisome proliferative activated receptor, delta (PPARD) (Marx, 2001), possibly as a result of inhibition of eicosanoid metabolism. PGI_2 is an activator of PPARD (Gupta et al., 2000; Lim et al., 2001), and the inhibition of carbaprostacyclin ($cPGI_2$)-stimulated DNA binding activity of the PPARD/RXR heterodimer is associated with induction of apoptosis in colorectal cancer cell (He et al., 1999). However, Piazza and colleagues reported a similar results with the sulindac sulfide related compound sulindac sulfone, which is

devoid of COX inhibitory activity (Piazza et al., 1997), suggesting that inhibition of PPARD was in part mediated by a direct, prostaglandin-independent effect (He et al., 1999).

Indomethacin induces nuclear receptor subfamily 4, group A, member 1 (NR4A1) (Kang et al., 2000), which induces apoptosis in a number of cell lines exposed to proapoptotic stimuli (Kuang et al., 1999; van Rensburg et al., 1999; Youn et al., 1999; Wilson et al., 2003). The induction of NR4A1 by indomethacin is associated with induction of apoptosis in HCT-15 colon cancer cells (Kang et al., 2000). Since these cells do not express COX-2, NR4A1 induction appears to be independent of COX-2.

NF-KappaB is a transcription factor which regulates the expression of a number of genes, including some that protect against cell death, such as the BCL and LAP family (Wang et al., 1998; Jones et al., 2000; Lee and Collins, 2001). NF-KappaB is held in the cytoplasm by I-KappaB until an appropriate signal results in its release. A variety of stimuli including cytokines such as TNF-alpha, IL-1, phorbol esters, LPS, viral infection, the human T-cell leukemia virus type 1-transforming protein Tax, ultraviolet radiation, and free radicals can result in the activation of I-KappaB kinases (IKKs). The IKKs add a phosphate group to I-KappaB which results in its degradation, freeing NF-KappaB. The NF-KappaB can then translocate to the nucleus to activate gene expression and stimulate cell apoptosis. Aspirin and other NSAIDs may exert their antiapoptosis effects by inhibiting the activation of the NF-KappaB pathway (Frantz and O'Neill, 1995; Marx, 2001; Tegeder et al., 2001; Yamamoto and Gaynor, 2001). The mechanisms by which NF-KappaB promotes cell survival are due in part to the up-regulation of anti-apoptotic genes such as members of BCL and LAP families (Wang et al., 1998; Jones et al., 2000; Lee and Collins, 2001). Also both aspirin and sodium salicylate can inhibit NF-KappaB by preventing I-KappaB phosphorylation and degradation (Pierce et al., 1996).

AP-1 is a group of related proteins consisting of products of the JUN, FOS, MAF and ATF subfamilies, which are activated in response to a number of stimulants including UV irradiation, growth factor, TNF-alpha and IL-1. Some of the genes regulated by AP-1 are involved in the immune and inflammatory response to tumour formation and progression, and promote proliferation and suppress apoptosis of tumour cells. AP-1 and NF-KappaB targeted genes partially overlap and most of these genes are activated by both AP-1 and NF-KappaB. Aspirin and COX-2 inhibitors have been shown to inhibit AP-1 activation, which would have an anti-tumour effect (Murono et al., 2000; Ding et al., 2003; Wong et al., 2004).

The WNT pathway is associated with carcinogenesis. WNT binds to membrane receptors encoded by Frizzled genes (FZD1-10). The canonical pathway involves WNT binding to FZD receptors, which leads to phosphorylation of the cytoplasmic protein Dishevelled (DSH), which then binds to axin and causes dissociation of the APC/axin/GSK complex, accumulation of beta-catenin and its subsequent translocation to the nucleus. There, beta-catenin inactivates gene transcription, some of it (e.g., c-Myc, cyclin DI) relevant to cancer. Aspirin and NSAIDs decrease the activity of WNT/beta-catenin pathway, although the precise mechanisms remain unclear (Dihlmann et al., 2003; Boon et al., 2004; Lu et al., 2005; Bos et al., 2006).

There is evidence that some carbonic anhydrase isozymes play a role in carcinogenic processes such as uncontrolled cell proliferation and malignant cell invasion (Kivela et al., 2005), and may be associated with a poor prognosis (Driessen et al., 2006) NSAIDs have been demonstrated to activate CA I and CA II isozymes in a dose-dependent manner (Puscas et al., 1996).

More recently, some studies have demonstrated that certain NSAIDs can inhibit cell cycle progression through inhibition of several kinases. The p70S6 kinase is a mitogen-activated kinase that is important for protein synthesis and G1 cell cycle

progression (Hashemolhosseini et al., 1998). Salicylate has been shown to inhibit the activation of p70S6 kinase, which results in a down-regulation of c-*myc*, cyclin D1, cyclin A, and proliferating cell nuclear antigen (Law et al., 2000), which play an important role in cell proliferation. Their down-regulation might contribute to salicylate-induced growth arrest. In human pancreatic cancer cells, it has been demonstrated that salicylate inhibits the progression from G1 to S and reduce cyclin D1 level (Law et al., 2000). The expression and activity of cyclin and cyclin-dependent kinases (Cdks) are also important in the progression of cell cycle, and aspirin or NSAIDs have been reported to inhibit them, blocking cell division.

NSAIDs have been reported to affect a number of other genes or pathways which could play a role in inhibiting tumour growth. The activity of ribosomal S6 kinase-2, involved in the activation of the mitogen-activated kinase cascade and the stimulation of cell proliferation and differentiation, is suppressed by NSAIDs (Stevenson et al., 1999). The COX-2 selective inhibitor, NS-398, has been reported to induce apoptosis in a number of colon cancer cell lines, including HT-29 (COX positive), HCT 15 (COX negative) and SW 480 (COX-negative) by releasing cytochrome c from mitochondria, leading to the activation of caspase-9 and caspase-3 (Li et al., 2002). Similar findings were reported by Ding (Ding et al., 2005). Exisulid, a derivative of the NSAID sulindac, which does not inhibit COX-2, has anticancer activity, inhibits cancer growth by inhibiting an enzyme that breaks down the intracellular messanger, cyclic GMP. Celecoxib can also induce apoptosis by blocking AKT activation independently of BCL-2 in human prostate cancer cells (Hsu et al., 2000), and by inhibiting (3-phosphoinositid-dependent kinase-1) PDK-1 activity in the HT-29 human colon cancer cell line (Arico et al., 2002). Growth differentiation factor 15 (GDF15), a member of the TGF-beta family of genes (Baek et al., 2001), has antitumourigenic and pro-apoptotic properties (Baek et al., 2001), and is up-regulated in human colorectal cancer cells by NSAIDs (Kashfi and Rigas, 2005).

This brief overview shows that the mechanisms by which NSAIDs exert their actions against cancers are potentially very complex. Although the detailed anticancer mechanisms of NSAIDs have not been fully elucidated, the effects of these compounds on cancer can be summarized as the inhibition of cell cycle progression (Shiff et al., 1995; Goldberg et al., 1996), the induction of apoptosis (Barnes et al., 1998; Giardina et al., 1999; Shao et al., 2000) and the inhibition of angiogenesis (Tsujii et al., 1998; Jones et al., 1999). The mechanisms by which NSAIDs inhibit tumourigenesis and progression are likely to be through a combination of COX-dependent and COX-independent pathways.

1.4. Evidence for COX-2 expression and effects of NSAIDs in oesophageal cancer

The link between the use of NSAIDs and a decrease in oesophageal cancer incidence has been demonstrated in both epidemiological and experimental studies. People who regularly used NSAIDs have a 40-50% decrease in death rate from oesophageal cancer in comparison with non-users (Thun et al., 1993; Funkhouser and Sharp, 1995; Farrow et al., 1998). Thun *et al.* (1993) found that subjects who used aspirin 16 times/month or more for at least 1 year had an approximately 40% lower risk of oesophageal cancer (p=0.054). The data from Funkhouser's study showed a 90% (95% CI=0.01-0.76) decrease in the risk of developing oesophageal cancer in subjects who were occasional aspirin users (Funkhouser and Sharp, 1995). The results of a large population-based case-control study (n=1144) showed a decreased risk of oesophageal adenocarcinoma (OR=0.37, 95%CI=0.24-0.58) and squamous cell carcinoma (OR=0.49, 95% CI=0.28-0.58) in users of aspirin relative to nonusers (Thun et al., 1993; Funkhouser and Sharp, 1995; Farrow et al., 1998). Apart from epidemiological evidence, experimental and clinical data suggest a possible preventative or therapeutic benefit of NSAIDs in oesophageal cancer. Li reported a significant inhibition of growth in 10 oesophageal cancer cell lines by ASA, which was time and dose dependent and was associated with induction of apoptosis (Li et

al., 2000). Langman showed the protective effects of NSAIDs against oesophageal cancer (Langman et al., 2000). In animal models of oesophageal carcinogenesis NSAIDS have reduced the frequency and number of premalignant and malignant lesions (Rubio, 1984; Rubio, 1986; Thun et al., 1993). Thus, there is significant evidence that NSAIDs can act as chemopreventive agents in oesophageal cancer.

As in other cancers, COX-2 mRNA, protein, or both, are up-regulated in oesophageal SCC and adenocarcinoma tissue or cell lines (Wilson et al., 1998; Zimmermann et al., 1999; Li et al., 2000; Kandil et al., 2001; Morris et al., 2001). Using an immunohistochemical staining technique, Ratnasinghe *et al* demonstrated strong positive staining for COX-2 in the well differentiated regions of squamous cell carcinoma of the oesophagus, and that smooth muscle cells, some stromal and inflammatory cells were also positive for COX-2 (Ratnasinghe et al., 1999). The same results were demonstrated in SCC of the oesophagus by reverse transcription-polymerase chain reaction (RT-PCR), Western blotting, and immunohistochemistry and immunofluorescence (Jiang et al., 2004). However, COX-2 expression has been shown to vary between SCC of the oesophagus from high-risk compared to low-risk areas, for reasons that are not known (Zhang et al., 2003). Increased expression of COX-2 in oesophageal adenocarcinoma has been found not only in the cancer cells themselves but also in the cells of the tumor stroma (Wilson et al., 1998; Zimmermann et al., 1999). In addition, increased expression of COX-2 mRNA and protein has been observed in premalignant conditions of the oesophagus, such as squamous dysplasia and Barrett's oesophagus (Wilson et al., 1998; Shamma et al., 2000; Kaur et al., 2002). The expression of COX-2 is progressively up-regulated through each of the stages of oesophageal carcinogenesis from Barrett's metaplasia through dysplasia to adenocarcinoma (Shirvani et al., 2000). Recent studies have also shown that COX-2 overexpression is related to cell proliferation in oesophageal squamous dysplasia and squamous cell carcinoma (Yu et al., 2003). This is similar to oesophageal adenocarcinoma where France has suggested that COX-2 expression might be a better prognostic indicator than

traditional histopathological staging (France et al., 2004). In a recent study conducted by Heeren *et al*, upregulation of COX-2 in adenocarcinoma of the oesophagus was associated with a poor outcome (Heeren et al., 2005).

The effects of NSAIDs have been studied in oesophageal cancer cell lines. The synthesis of PGE_2 is increased in cells expressing COX-2 compared to those cells expressing COX-1 only (Zimmermann et al., 1999). Li reported that the inhibition of growth by aspirin in 10 oesophageal cancer cell lines was dose- and time-dependent, and was associated with induction of apoptosis (Li et al., 2000). Recently Cheong *et al* demonstrated that synthetic and naturally occurring COX-2 inhibitors suppressed proliferation, and induced apoptosis and cell cycle block, in human oesophageal adenocarcinoma cells (OE33) *in vitro* (Cheong et al., 2004).

COX-2 expression can be induced in oesophageal tissues by the tobacco carcinogen benzo[a]pyrene diol epoxide (BPDE) and by tumour promoting bile acids (Zhang et al., 1998; Li et al., 2000; Song and Xu, 2001). These data demonstrated the relationship between COX-2 and oesophageal cancer, but its potential role in cancer development and progression needs further investigation.

1.5. The aims of this study

Epidemiological and experimental studies have shown that the use of NSAIDs can significantly reduce the risk of and the mortality from a variety of cancers, including oesophageal cancer. Experimental studies on oesophageal cancer cell lines expressing COX-2 have shown pro-apoptotic effects of NSAIDs. Although COX-2 and its products, prostaglandins, have been reported to be important in tumourigenesis, the mechanisms are not certain. It is not clear if it is the upstream regulators of COX-2 that are affected by NSAIDs, or whether the effects of these compounds on oesophageal cancer are by COX-2 independent pathways. Reported studies are based on cell lines *in vitro* and rodent animal experiment. No effects of

NSAIDs on human oesophageal cancer tissues have been reported.

For this thesis the incidence of and surgical outcomes from oesophageal cancer were reviewed, based on the published data from the Department of Thoracic Surgery, the Fourth Hospital, Hebei Medical University. Then the expression of COX-2 in oesophageal cancer tissue was measured, and the effects of non-selective and selective COX-2 inhibitors on an oesophageal cancer cell line expressing COX-2 was determined. To determine the clinical relevance of these studies, the effects of NSAIDs were measured on oesophageal cancer tissue *in vivo*. Then a small clinical trial of aspirin was established to determine if it might increase survival in patients who have had an oesophagectomy. All experimental and clinical work described in this thesis was carried out in Fourth Hospital, Hebei Medical University, China.

Chapter 2. Materials and methods

2.1. Immunohistochemistry on paraffin embedded tissue

Immunohistochemistry was performed using an avidin-biotin peroxidase complex technique, essentially as described by Sano (1995). Five micron thick sections were cut from paraffin blocks, dewaxed in xylene, and then rehydrated in absolute alcohol. Endogenous peroxidase activity was blocked with 0.5% hydrogen peroxidase in methanol for 30 minutes. Antigen retrieval was performed by heating the sections for 5 min in 10 mM citrate buffer (pH 6) in a microwave oven. Non-specific binding was blocked with 3% normal horse serum. The primary antibody, an affinity-purified rabbit polyclonal anti-human COX-2 IgG (Cayman Chemical, USA), was used at a dilution of 1:60. The secondary antibody was a biotinylated goat anti-rabbit IgG (Zymed Laboratories Inc. South San Francisco, USA), with diaminobenzidine as the chromogen. Sections were lightly counter-stained with Meyer's haematoxylin, dehydrated and mounted on glass slides. Primary antibody was omitted as a negative control, and colon cancer tissue was used as a positive tissue control.

Each slide was scored by two pathologists blind to the clinical or pathological outcomes of the patients. Staining was graded according to the percentage of COX-2 positive cells: grade 0 had no positive cells, grade 1 had less than 10%, grade 2 10% to 50%, and grade 3 more than 50% positive cells. In each section 5 high-power fields were selected randomly for assessment. The intracellular location and pattern of COX-2 staining, and the distribution of COX-2 positive cancer cells within the tumour, were also recorded.

2.2. Measurement of expression of COX-2 or PCNA by flow cytometry

For paraffin embedded tissues, 40-50 μm sections were cut and de-waxed in dimethylbenzene at room temperature for 48 hours, and then rehydrated through graded ethanols before trituration. Fresh tissues were fixed in 70% ethanol and stored at 4^{o}C until they were cut into small pieces (about 1 mm^{3} in size) with scissors before trituration. The method of carrying out flow cytometry on cells from paraffin-embedded tissues has been validated and used for many years in the author's institution (Zuo et al., 1987; Zuo, 1988b; Du et al., 2004).

Cell suspensions were prepared by trituration through a bronze mesh (120 holes per square inch) to remove tissue fragments and cell clusters. The samples were then filtered through a finer sieve (200 holes per square inch). The cell suspensions were centrifuged at 100 g for 3 minutes and the cell pellets washed twice with PBS. Finally the pellet was re-suspended in PBS to a concentration of 1×10^{6} cells per milliliter.

The expression of COX-2 in cells from paraffin embedded tissue and PCNA in fresh tissue was measured by flow cytometry. First 100 μl suspensions of the cells were aliquoted into polystyrene tubes and incubated with 100 μl of FITC-conjugated mouse anti-human COX-2 antibody (Cayman Chemical, USA) or 100 μl of FITC-conjugated mouse anti-human PCNA antibody (Zhongshan Biotechnology Co. Ltd, Beijing, China) diluted 1:100, for 30 minutes at 37^{o}C. The labelled suspensions were washed with PBS and centrifuged at 100 g for 5 minutes. The supernatant was removed and the cell pellet was re-suspended in 1 ml of PBS, and then filtered through a sieve (500 holes per square inch).

As a negative control, cells were incubated with PBS in the place of the FITC-conjugated mouse anti-human antibody. As an isotype control,

FITC-conjugated rabbit serum was used. Tissues from the cancer free margins were used as normal tissue controls.

All samples were analyzed using a FACS420 flow cytometer (Becton Dickinson, Sunnyvale, California, USA). The cells were excited with a single 488 nm argon laser. Fluorescein fluorescence was detected through a 520 nm band pass (BP) filter. At least 10,000 events were collected for each sample, and electronic gates were used to subtract the background fluorescence of the isotype controls. The obtained fluorescence signals were recorded as logarithmically amplified data. Data analyses were performed using Single Histogram Statistic software (Becton Dickinson) in a HP-300 Consort 30 computer. Before the analysis of the flow cytometry data, chicken red blood cells were used as a standard for calibrating the coefficients of variation (CV) of the instrument to less than 5%.

The number of COX-2 or PCNA positive cells in a cell suspension was determined by an arbitrary threshold setting which allowed no positive counts in the isotype control, and was expressed without subtraction of the non-specifically stained fraction. The Fluorescence Index (FI) was calculated as the ratio of fluorescence intensity of the sample with subtraction of fluorescence intensity in background compared to mean fluorescence intensity of normal controls. The mean FI plus or minus 2 standard derivations in normal controls was defined as normal expression, and values higher than the upper limit were regarded as positive for the expression of the antigen. The positive rate of COX-2 or PCNA expression was defined as the ratio of the number of COX-2 or PCNA positive cancers to the total number of studied cancers.

The FI values in the primary cancers, metastatic lymph nodes and normal tissues were compared using the Student's *t* test. The correlation between positive expression of COX-2 and clinicopathological factors were assessed with Chi-square test. Survival time was calculated from the day of surgery to death or until

November 20, 2000 with a maximum follow-up of 199 months (mean follow-up, 66 months). The survival rates of patients grouped according to the FI values of their oesophageal cancers were calculated with Kaplan-Meier method; statistical significance was assessed using log-rank test. The expression of PCNA in cancer tissue from meloxicam users and non-users was compared using Student's t test. All statistical analyses were performed with the use of a SPSS software package (SPSS 9.0, Inc., Chicago, IL). The differences were considered to be significant when the P value was less than or equal to 0.05.

2.3. Culture of the TE-13 cell line

The human oesophageal SCC cell line TE-13 was cultured in RPMI-1640 medium (Gibco BRL, USA), supplemented with 10% foetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin, at 37^{o}C in a humidified atmosphere of 95% air and 5% CO_2. The cells, seeded into 96-well flat bottom tissue culture plates at a density of 10^4 cells per well, were incubated for 24 hours to allow them to adhere. Before the addition of drug, they were washed and incubated for 30 minutes in fresh medium without FBS. The cells were then cultured in medium containing aspirin at concentrations of 0 (control), 1, 4, or 8 mmol/L, or containing NS-398 (Sigma, USA) at concentrations of 0 (control), 0.001, 0.01, 1, 100 μmol/L for varying periods of time before being harvested.

2.4. Measurement of inhibition of cell proliferation by MTT reduction

Cell proliferation in cultured TE-13 cells was measured in an MTT reduction assay. A tetrazolium salt, 3- (4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), was added, to a final concentration of 0.5 mg/L, to cells that had been incubated for 72 hours with or without drug. After incubation for 4 hours at 37^{o}C the supernatant was removed, 150 μl DMSO was added to each well, and the culture plate was shaken for 10 minutes. The absorbance was then measured with a dual

wavelength automatic reader at 490 nm. Inhibition of proliferation was calculated as the absorbance of the culture with aspirin or NS-398 divided by the absorbance of the culture with no drug, expressed as a percentage.

2.5. Measurement of cell apoptosis

Both floating and attached cells were harvested from TE-13 cell cultures. The attached cells were harvested with trypsin/EDTA. The cells from fresh tissue were seperated according to 2.2. The harvested cells were washed twice with phosphate buffered saline (PBS). The cells were fixed in 70% ethanol and then stained with 1 ml of propidium iodide (PI), at a final concentration of 50 μg/ml, for 20 min. Flow cytometric analysis was performed on a FACS420 flow cytometer. The method for calculating the apoptotic rate is described in more detail in Section 6.3.6 and Figure 6.11.

2.6. Electron microscopy

Cells were examined by transmission electron microscopy. Cultured TE-13 cells were treated with 2.5% trypsin, and the cells were collected by centrifugation at 1000 rpm for 10 minutes and washed twice in PBS. The cell pellet was fixed with 2.5% glutaraldehyde for at least 12 hours at 4^{o}C. The fixed cells were washed twice with phosphoric acid buffer for 10 minutes each time, dehydrated in a graded series of ethanol after postfixation in 1% osmic acid, and washed in distilled water 3 times. The samples were embedded in Eponresin 812 (Araladite, Warrington, PA, USA). Tissues were cut into cubes approximately 1 mm^3 in size and immediately fixed in 4% glutaraldehyde. After postfixation in 1% osmium tetroxide the samples were embedded in Eponresin 812.

Ultrathin sections were cut with an ultrotome and stained with uranyl acetate and lead citrate. Sections were examined with a Hitachi H-600 transmission electron microscope (Hitachi, H-600, Tokyo, Japan).

2.7. Measurement of prostaglandin 6-keto-PGF1α by radio-immuno-assay

The concentration of 6-keto-PGF1α in the supernatant of cultured TE-13 cells, and in tissues and blood from patients who used meloxicam, was measured with RIA. Cell culture supernatants were collected and centrifuged to remove cells and debris. Venous blood (5 ml) was collected into a syringe containing 0.1 ml indomethacin-Na_2EDTA as anticoagulant, and then centrifuged at 3,500 rpm for 15 minutes at 4^{o}C. The plasma was collected and stored at -20^{o}C until analysis. Five to ten grams of tissue sample were homogenized in 0.1 ml of 100% ethanol, and homogenization continued after 0.9 ml normal saline was added. The sample was centrifuged at 3500 rpm for 15 minutes at 4^{o}C before removal of the supernatant. The precipitate was stored at -20^{o}C until analysis. The concentration of 6-keto-PGF1α in the preparations was measured by radio-immunoassay using a commercially available kit (Beifang Biotechnology Co, Beijing, China).

2.8. Reverse transcription PCR

RT-PCR was used to measure COX-2 gene expression in fresh tissues and TE-13 cells. Total RNA was isolated using TRIzol reagent following the protocols of the manufacturer (Gibco BRL). An aliquot of this was reverse transcribed into cDNA using an RNA PCR-Kit (Sino-American Biotechnology, Beijing, China). The PCR protocol was an initial 94^{o}C for 5 minutes, followed by 40 cycles at 94^{o}C for 1 minute, 55^{o}C for 1 minute, and 72^{o}C for 1 minute. Human specific primers were used for COX-2 and beta-actin. The primers used for the COX-2 amplification were 5′-TTC AAA TGA GAT TGT GGG AAA ATT GCT-3′ (forward primer) and 5′-AGA TCA TCT CTG CCT GAG TAT CTT-3′ (reverse primer), giving a 303 bp PCR product. For beta-actin (ACTB), the forward primer was 5′-GTT TGA GAC CTT CAA CAC CCC-3′, and the reverse primer was 5′-GTG GCC ATC TCC TGC TCG AAG TC -3′, giving a 318 bp PCR product. The synthesised PCR products were separated on

1.5% agarose gels and analysed by Gel-Pro analyser version 3.1 software. The ratio of COX-2 to beta-actin was used for expressing the relative level of mRNA expression.

2.9. Protein extraction and Western blotting

Protein was extracted by homogenising the cells in a lysis buffer containing 100 mmol/L 4- (2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES）, 10 mL/L Triton X-100, 1 mmol/L ethylenediaminetetraacetic acid (EDTA), 10 mmol/L dithiothreitol (DTT), and 1 mmol/L phenylmethylsulphonylfluoride (PMSF) (Promega, USA) and incubating on ice for 15 minutes. The lysate was centrifuged at 20,000 g for 15 minutes, the supernatant collected, and its protein concentration determined by Coomassie blue (CBB G250 assay kit, Nangjing Jiancheng Biotechnology Research Institute). Then, 100 μg of protein was separated on a SDS-10% polyacrylamide gel and transferred electrophoretically to a nitrocellulose membrane. After the non-specific binding sites were blocked with 2% dried skim milk in TBS (120 mM Tris HCl, pH 7.4 and 150 mM NaCl), the membrane was incubated overnight with 2 μg/mL rabbit antibody to COX-2 or I-KappaB (Santa Cruz, USA). After washing with 0.05% Tween 20 in TBS, and the membrane was incubated with a horseradish peroxidase-conjugated goat antirabbit developed with diaminobenzidine. The immune complex was visualised and quantified with Gel-Pro analyser software (Media Cybernetics, USA), and the results expressed as units of integrated optical density (IOD).

2.10. Electrophoretic mobility shift assay (EMSA)

An electrophoretic mobility shift assay (EMSA) was used to detect the expression of NF-kappaB in tissues or cultured cells. A sample of the tissue (15-20 mg) or cultured TE-13 cells (1×10^6) were homogenized in 1 ml of buffer (Buffer A: 10 mmol/L HEPES, 1.5 mmol/L $MgCl_2$, 10 mmol/L KCl, 0.5 mmol/L dithiothreitol (DTT), 0.5mmol/L, phenylmethylsulphonylfluoride (PMSF); 0.1% Nonidet P40

(NP-40), pH 7.9) and centrifuged at 1,000g for 10 minutes at 4°C. After the supernatant was removed, the pellet was resuspended in 1 ml of buffer A without NP-40. The samples were centrifuged at 1000g for 10 minutes at 4°C, and the supernatant was removed. Then 0.1 ml of buffer (Buffer B: 20 mmol/L HEPES, 0.42 mmol/L NaCl, 1.5 mmol/L $MgCl_2$, 0.2 mmol/L EDTA, 0.5 mmol/L DTT, 0.5 mmol/L PMSF, 2.5% glycerol, pH 7.9) was added to the sample. It was shaken vigorously and incubated for 1 hour at 0°C. Finally the samples were centrifuged at 15,000g for 15 minutes at 4°C, and the supernatant which contained the nuclear extract was retained and stored at -80°C.

The protein concentration was determined using the CBB G250 assay kit. Double-stranded deoxyoligonucleotides containing the NF-kappaB consensus recognition site (5'-AGT TGA GGG GAC TTT CCC AGG-3'; Promega, USA) were end-labelled with [γ-32P]ATP using T4 polynucleotide kinase (Promega) and purified by ethanol precipitation. A 26-bp oligonucleotide harboring the consensus AP-2 binding element （5'-GAT CGA ACT GAC CGC CCG CGG CCC GT-3'）(Promega) was used as the unlabeled competitor. The nuclear protein (10 μg) was incubated with the radiolabelled probe DNA (3.5 pmol, 10 μCi), with or without a 100-500 fold excess of unlabelled probe, in 1 mmol/L $MgCl_2$, 0.5 mmol/L EDTA, 0.5 mmol/L DTT, 50 mmol/L NaCl, 10 mmol/L Tris-HCl (pH 7.5), 0.05 μg poly (dI-dC), and 40 ml/L glycerol to a final volume of 10 μl, for 30 min at room temperature. The DNA-protein complexes were resolved on a 6% non-denaturing polyacrylamide gel which was then dried and visualized by autoradiography at -80°C.

2.11. Ethics approvals

Studies involving patients and human tissues were carried out in accordance with the requirements and approval of the Fourth Hospital, Hebei Medical University, China.

Chapter 3. Review of epidemiological and surgical studies of oesophageal cancer in Hebei, China

3.1. Introduction

Oesophageal cancer is the seventh most common malignancy worldwide (Parkin et al., 1993), the fourth most frequent cancer in China (Li et al., 1996), and the leading cause of cancer death in Hebei Province, China (Hou et al., 1995). In the southern area of Hebei Province, the incidence of squamous cell carcinoma (SCC) of the oesophagus is reported to be the highest in the world, at 199 cases per 100,000 population (Hou et al., 1995).

From the 1970s to the 1990s three large scale epidemiological studies of oesophageal cancer were carried out in Hebei Province of China. The findings were only published in Chinese. The Fourth Hospital of Hebei Medical University, is the major cancer hospital serving this region. From the establishment of the Department of Thoracic Surgery in September 1952 to August 2002, 17,149 oesophageal resections for cancer of the oesophagus or gastric cardia have been performed (Ping, 2002). While the experiences of this Department have been reported at different periods during this time, most of the reports were in Chinese.

In this Chapter, articles reporting epidemiological studies published during the past half century in Hebei, and all articles on surgery for carcinoma of the oesophagus and gastric cardia performed in the Department of Thoracic Surgery, Fourth Hospital, Hebei Medical University between 1952 and 2000 are reviewed. The articles for analysis were selected on the following criteria: publications in a national journal or reported at a national meeting; all publications on the three epidemiological investigations; surgery for cancer of the oesophagus or cardia, which was confirmed

histopathologically; a minimum of 1,000 patients in the report; the number of patients, postoperative mortality and morbidity was detailed.

3.2. Epidemiology of oesophageal cancer in Hebei Province, China

In the 1960s surgeons noted that many patients with cancer of the oesophagus and gastric cardia were from the southern areas of Hebei Province. For this reason, three large-scale epidemiological investigations on oesophageal cancer were carried out with the support of Chinese Health Ministry and Hebei Provincial Department of Public Health in the 1970s (Hou et al., 1995), 1980s (Hebei, 1980) and 1990s (Ma et al., 1989). In each of the three investigations, oesophageal cancer was the foremost reason for cancer deaths in Hebei Province. The results also revealed that the incidence of oesophageal cancer decreased in males from 41.5/10,0000 in the 1970s to 36.39/10,0000 in the 1990s. The same trend was seen in females: from 19.45/10,0000 in the 1970s to 19.25/10,0000 in the 1990s. In regard to the geographical distribution of oesophageal cancer, the incidence was found to gradually decrease from south to north in Hebei. The highest incidence area of oesophageal cancer was in southernmost Cixian county, at 125.34/100,000 for males and 69.25/100,000 for females, while the lowest occurrence region was in northern Hebei's Chicheng County, with an incidence of 5.1/100,000 in male, and 1.4/100,000 in female (Hou et al., 1995) (Figure 3-1).

In the early 1970s, amongst the reasons identified for the high incidence of oesophageal cancer were a high concentration of nitrosamines in the drinking water and pickled vegetables, contamination of foodstuff by fungi (most commonly *Aspergillus flavus*), and a lack of selenium, zinc and/or molybdenum in the soil of the high incidence areas. Since then great efforts have been made to improve the water-supply system (drinking water has been changed from well water to tap water) and the methods for storing grain (e.g., properly drying the grain in the sun).

Fertilizers containing selenium, zinc and molybdenum have been used in the high incidence area of oesophageal cancer. At the same time, Vitamin B2 and the Chinese medicines, Zeng Sheng Ping and Cang Dou Wan (Hou et al., 1996), which have been shown to suppress epithelial proliferation, have been administered to the high-risk population. All of these measures may have contributed to the decrease in oesophageal cancer occurrence in the high-incidence area of Hebei.

3.3. Diagnosis of oesophageal cancer

A variety of methods have been used in the diagnosis of cancer of the oesophagus and gastric cardia over the years. Oesophagraphy is extensively used in almost all patients, usually as a double-contrast technique, particularly in patients with suspected early oesophageal cancer. Before the 1990s, balloon cytology was used to obtain specimens for cytology in mass screening programmes for oesophageal cancer, especially for early cancers (Figure 3-2) (Department, 1967; Shen and Wang, 1998). From 1980 onwards fiberoptic oesophagoscopy has been employed in the diagnosis of cancer of the oesophagus and gastric cardia, and progressively for population screening after 1990. Mucosal staining with iodine or methylene blue is used in the diagnosis of early oesophageal cancer to direct from which part of the oesophagus biopsies should be taken (Figure 3-3).

In recent years, computed tomography (CT) has been used to exclude the involvement of adjacent organs and to identify metastases. Aortic invasion is assumed if more than 90^{o} of the peri-aortic low-density layer is not apparent. Bronchoscopy has also been used to assess invasion of the trachea or the main stem bronchus, when polypoid intrusion into the tracheobronchial lumen or marked airway deformity has been identified by CT.

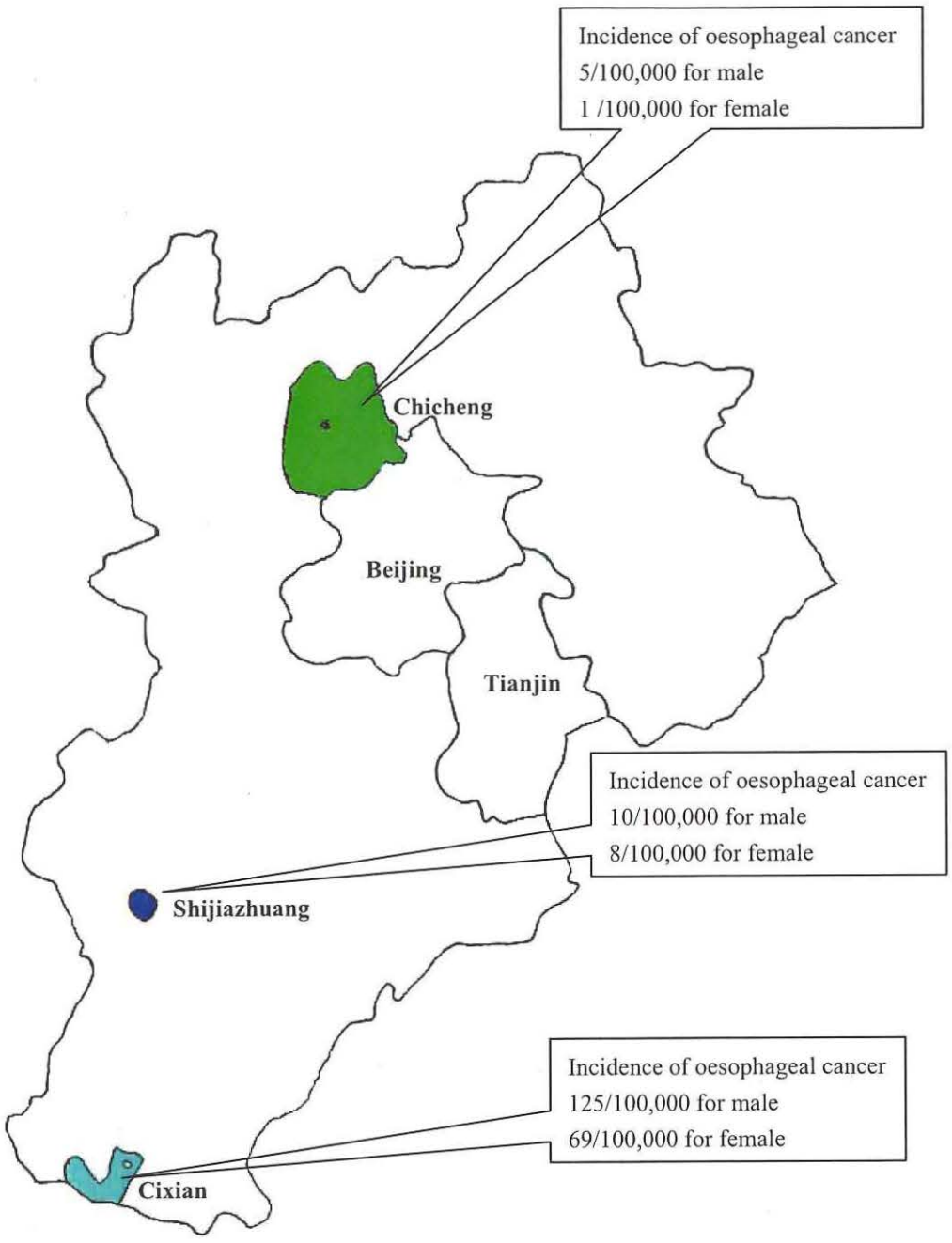

Figure 3-1. The incidence of oesophageal cancer in different regions of Hebei Province, China (Hou, et al., 1995)

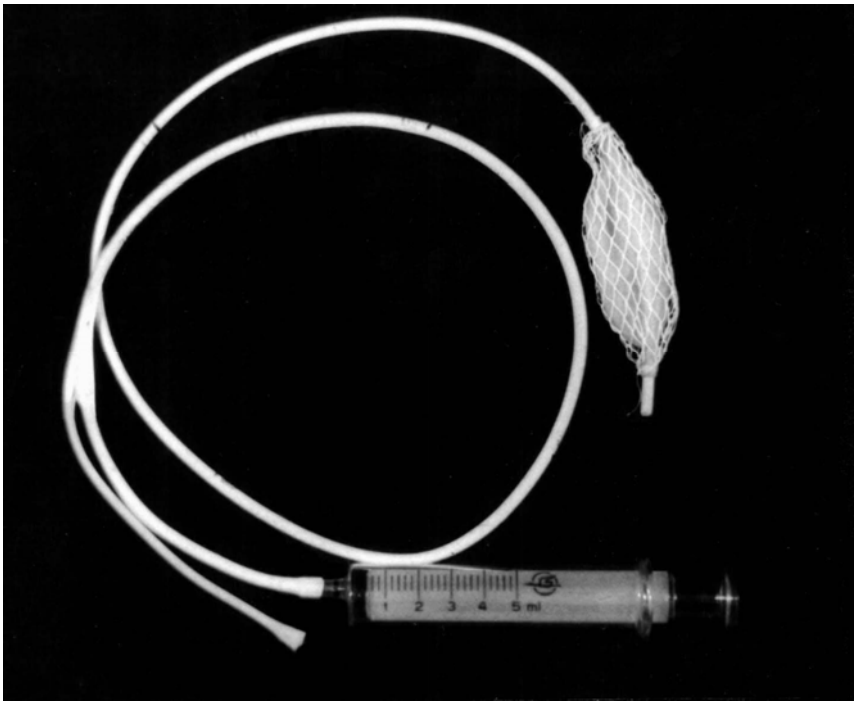

Figure 3-2. The balloon apparatus for collecting cell samples from the oesophagus, with the syringe in place.

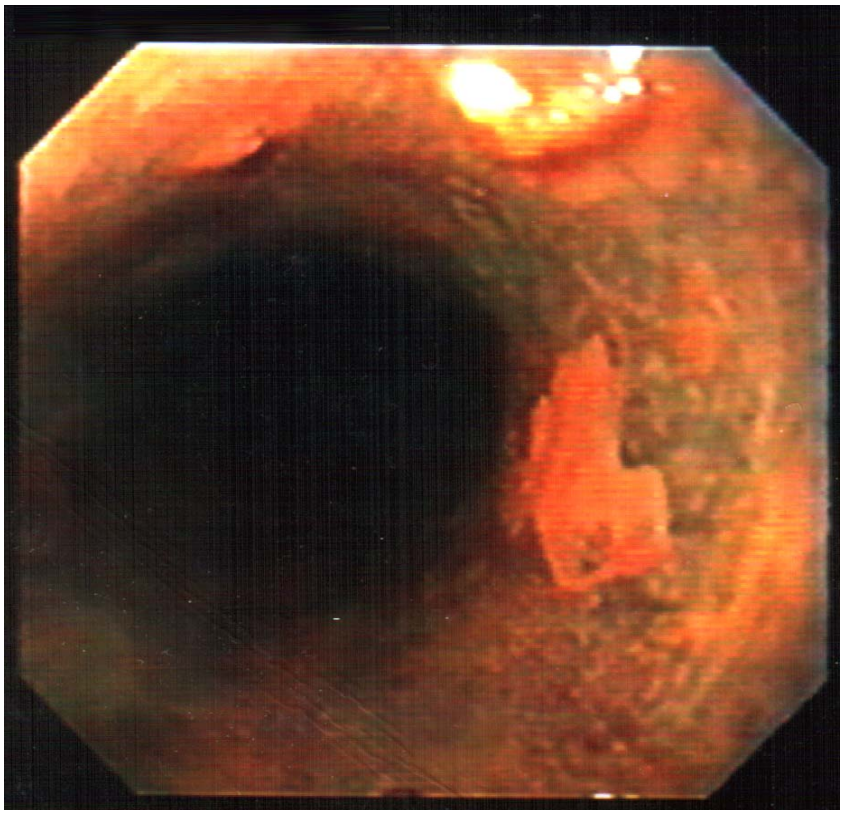

Figure 3-3. Lugol's staining for a superficial oesophageal carcinoma stained with Lugol's iodine. The normal oesophageal mucosa stains dark brown while the cancer area remains unstained.

3.4. Surgical technique

A left posterolateral thoracotomy is used for cancers in the middle and lower third of the oesophagus and in the cardia. Efforts are made to remove all paraoesophageal and cardiac lymph nodes. According to the criteria proposed by The Chinese Anti-Cancer Association, the longitudinal resection margin is more than 5 cm from the tumour (Huang, 1991). Oesophagogastrostomy is usually performed in the thorax (Figure 3-4; Figure 3-5). For the cancers located in the upper third of the oesophagus, the oesophagus and stomach are mobilized and resected through a left posterolateral thoracotomy, with oesophagogastrostomy in the neck (Figure 3-6). Trans-hiatal oesophagectomy without thoractomy is used only in patients with tumour confined to the oesophageal wall and compromised cardiopulmonary reserve (Figure 3-7). A combined thoracoabdominal incision is employed occasionally for advanced cancers of the gastric cardia and a laparotomy-only approach is frequently used in patients with cancer in the gastric cardia, especially those with compromised cardiopulmonary function.

In the majority of patients oesophagogastrostomy following oesophageal resection is usually performed by open anastomosis with direct suture in 2 layers (Figure 3-8). Anastomosis is performed occasionally using a circular anastomotic stapler (end-to-end anastomosis) (Figure 3-9). A sutureless oesophagogastric anastomosis using an intraluminal elastic circular ligation was first reported by the Department of Thoracic Surgery, Fourth Hospital, Hebei Medical University, China (Figure 3-10). The basic principle in this technique is to use an elastic ring to fix the oesophageal and the gastric walls around a supporting tube, which has been placed in the oesophageal cavity. The distal tissue beyond the elastic rings gradually necroses because of ischemia, and finally separates from the normal tissue. The supporting tube with the elastic ring and the necrotic tissue falls from the anastomotic site into the stomach, and is then absorbed or excreted from the alimentary tract.

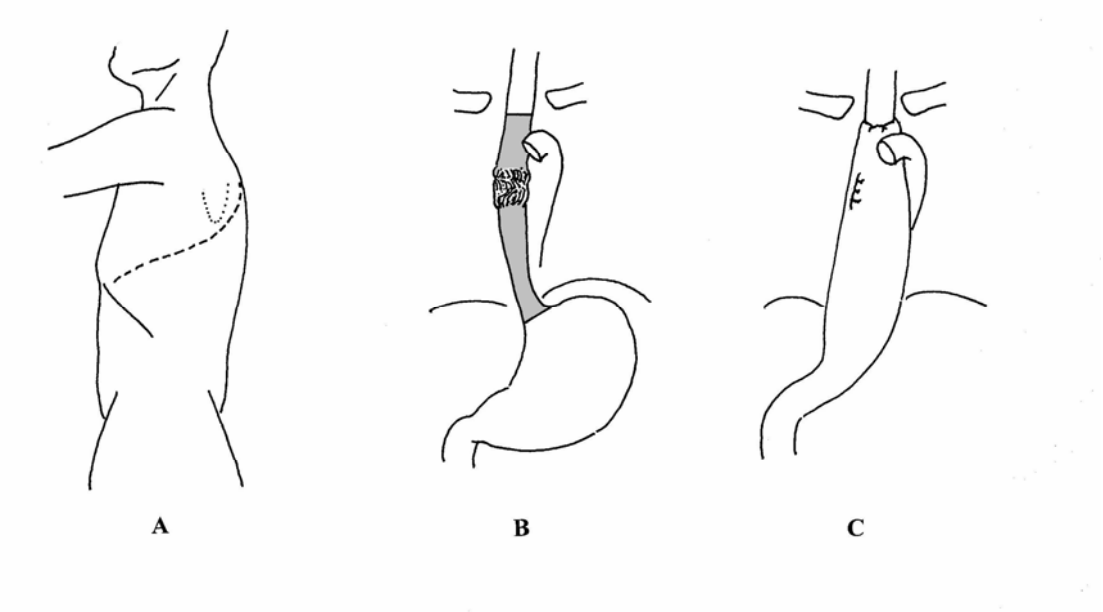

Figure 3-4. Technique of oesophagectomy and oesophagogastrostomy for carcinoma of the middle oesophagus. (A) Site of incision (dashed line). (B) Extent of resection (shaded area). (C) Completed oesophagogastrostomy. The anastmotic site is above the aortic arch.

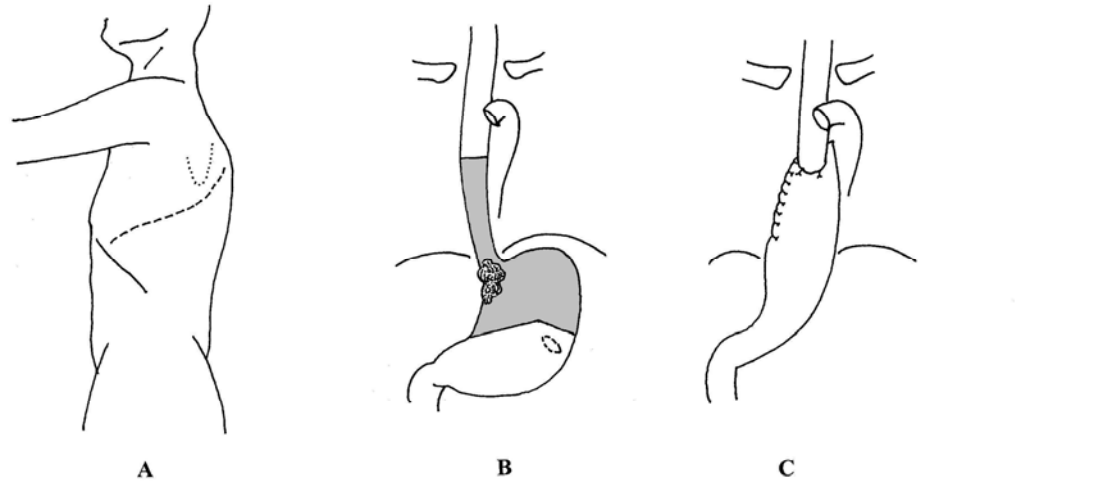

Figure 3-5. Technique of oesophagogastrectomy and oesophagogastrostomy for cacirnoma of the gastric cardia. (A) The site of incision (dashed area). (B) Extent of resection (shaded area). (C) Completed oesophagogastrostomy. The anastomotic is under the aortic arch.

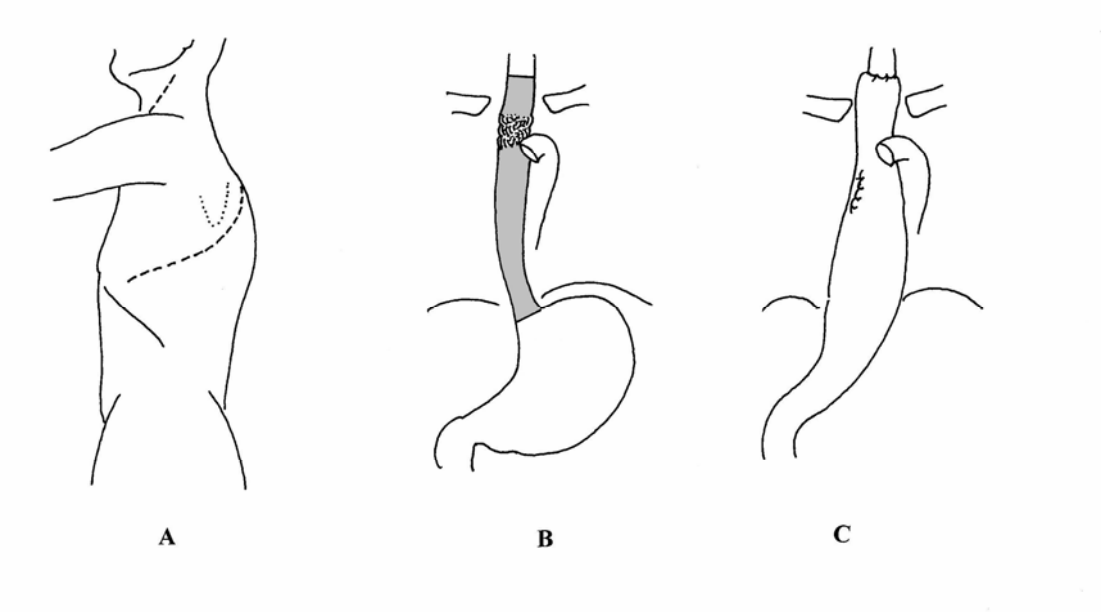

Figure 3-6. Technique of oesophagectomy and oesophagogastrostomy for carcinoma of the upper thoracic oesophagus. (A) Site of incision (dashed line). (B) Extent of resection (shaded area). (C) Completed oesophagogastrostomy. The anastomotic site is in the neck.

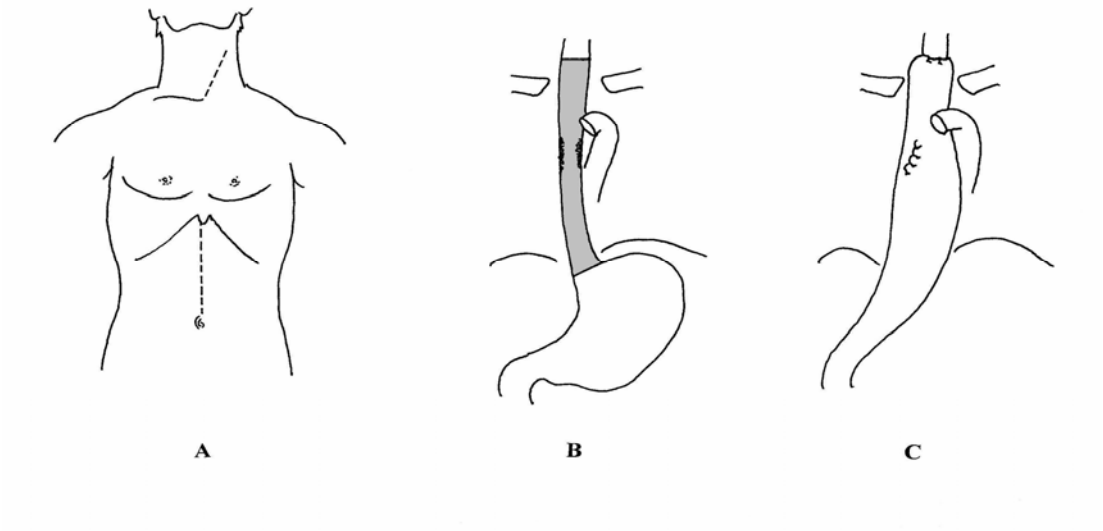

Figure 3-7. Transhiatal oesophagectomy without thoracotomy. (A) Upper abdominal midline and cervical incision (dashed lines). (B) Extent of resection (shaded area). (C) Completed anastomosis. The anastomotic site is in the neck.

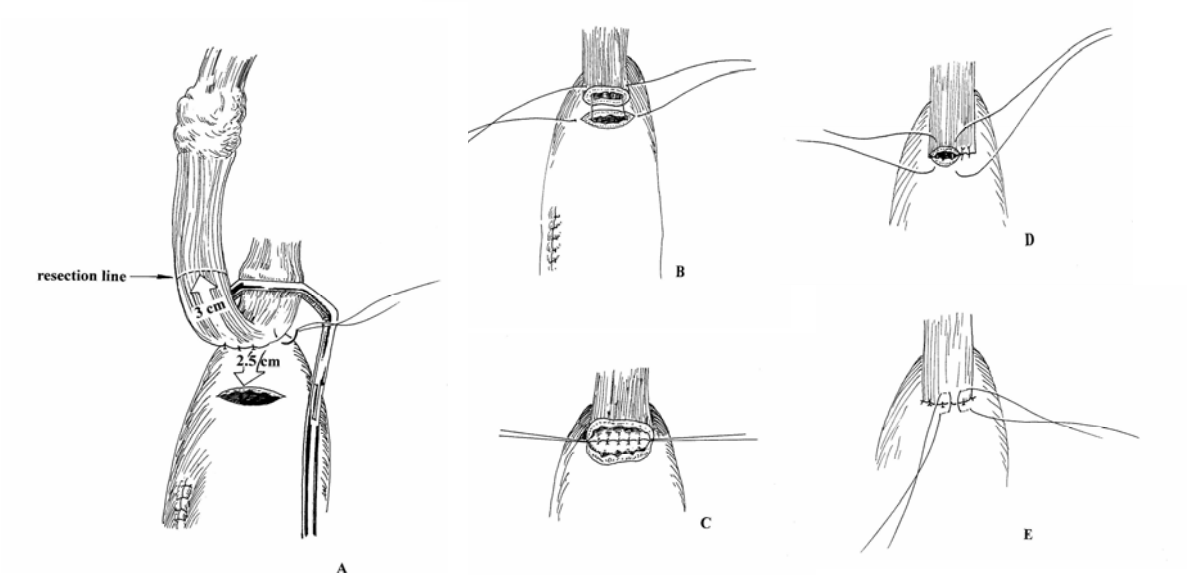

Figure 3-8. Oesophagogastric anastomosis with hand sewing. (A) After an incision is made on the fundus of the stomach, the outer posterior layer of the anastomosis is carried out by suturing fibromuscular layer of the oesophagus to the seromuscular layer of the stomach with a fine silk placed in horizontal mattress fashion. (B, C) After excision of the specimen, the inner posterior layer of the anastomosis is performed and completed by interruptedly suturing full thickness of the oesophagus and stomach. (D) Anterior inner layer of the anastomosis is performed by approximation of full layers of the oesophagus and the stomach with interrupted fine silk sutures. (E) The anterior outer layer of the anastomosis is finished by approximating the fibromuscular layer of the oesophagus to the seromuscular layer of the stomach with a mattress suture of interrupted silk.

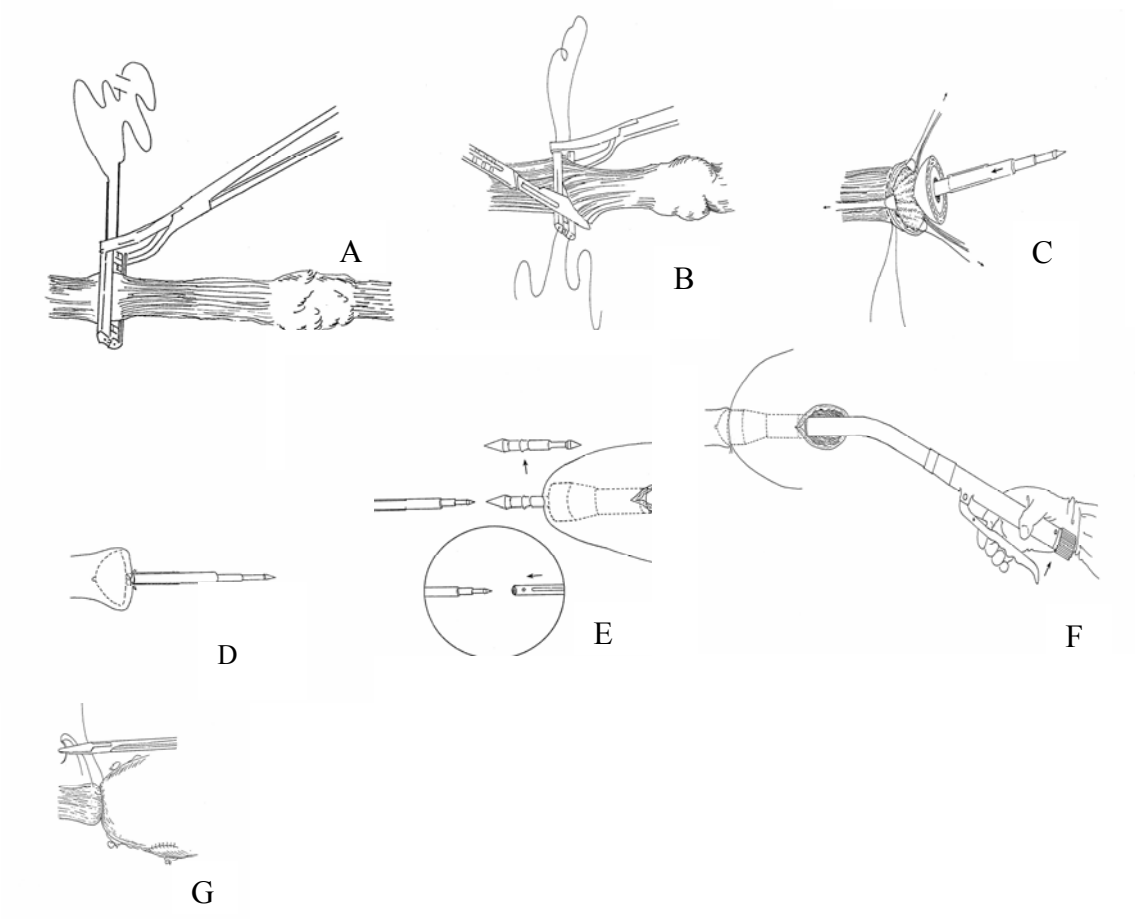

Figure 3-9. Oesophagogastric anastomosis with circular anastomotic stapler. After a purse string is placed (A), the oesophagus is transected (B). The anvil is put into the lumen of the oesophagus (C) before the purse string is tightened (D). The circular stapler is applied through a gastrotomy (E), and then the gasket is fired (F). After removal of the instrument, the anastomosis is strengthened by suturing the fibromuscular layer of the oesophagus and seromuscular layer of the stomach.

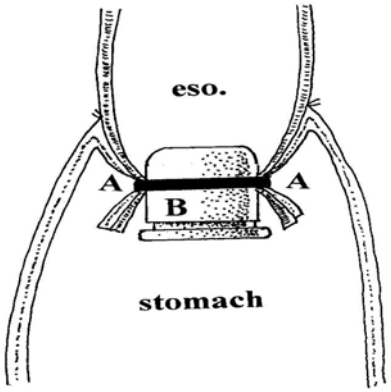

Figure 3-10. Schematic diagram of oesophagogastrostomy by intraluminal elastic circular ligation. A. elastic ring; B. supporting tube.

3.5. Resectability

In the past 50 years, the resectablility rate for cancer of both the oesophagus and the cardia has increased gradually, although it has always been slightly lower for cancer of the cardia compared to the oesphagus (Table 3-1). This improvement has mainly been due to an increasing proportion of early cancers being diagnosed early, which outweighs the exclusion of patients with unfavourable tumours as identified by CT. It is also likely that surgical techniques and supportive care have improved, which has allowed resection in patients previously considered unfit for surgery. We reported a group of 141 patients with early cancer of the oesophagus and cardia, in which the resectability rate was 100% (Liu et al., 1995). We also analyzed the relationship between tumour site and the resectability rate in 1982. In the oesophagus, the resectability rate was higher for lower-third tumours (87%) than for lesions of the middle (77%) and the upper thirds (66%) (Zhang et al., 1982).

3.6. Postoperative complications and deaths

The incidence of major postoperative complications has gradually decreased over time (Table 3-2). This probably results from improvements in preoperative

preparation, postoperative management, surgical and anaesthetic techniques, patient's nutritional status, availability of effective antibiotics, and the establishment of an intensive care unit. Before the 1970s, for example, hypoproteinaemia was common at the time of admission owing to poor nutritional intake. The average operating time was over 4 hours, and there was no intensive care unit for postoperative support.

The most common complication was anastomotic leakage. The incidence of anastomotic leakage was reduced from 5% between 1952 and 1976 to 3.4% between 1984 and 1989 (Adachi et al., 1996). This was not associated with the technique used; the leak rate was 3.4% for hand-sewn anastomoses, 2.7% for stapled anastomoses and 2.7% after elastic ligation. The reasons for the leakage have been found to be high tension and local infection at the anastomotic site, and oedema and ischaemia of the gastric wall (Ping et al., 1982). It remains difficult to distinguish between anastomotic leakage and perforation of the stomach caused by ischaemia (Ping et al., 1982). In general, anastomotic leakage in the neck did not result in severe infection and malnutrition, and fasting was not necessary for these patients. The area of leakage was dressed, applying mild local pressure to prevent the further leakage of swallowed food and gastric juice. In contrast, intrathoracic leakage usually led to severe infection, fluid imbalance and malnutrition. Treatment usually involved adequate control of infection, thorough drainage of the thoracic cavity, maintenance of nutrition, and correction of fluid imbalance. Although parenteral nutrition has been used increasingly in later years, the preferable current method is a feeding jejunostomy. Reoperation was performed for 11 leaks before the 1980s (7 anastomotic leaks and 4 perforations of the stomach), and anastomosis was performed again either in the neck or in the uppermost thorax. However, only one patient recovered, and leakage occurred again in the remaining 10, resulting in 3 deaths (Ping et al., 1982). Reoperation for anastomotic leakage was abandoned after this time.

Some specific complications of oesophageal resection merit particular comment.

Chylothorax was an uncommon complication, with an incidence of less than 1.5 % (Table 3-2), but its management is always a challenge (Alexiou et al., 1998; Merigliano et al., 2000). Chylous output of less than 1,000 ml/day was managed conservatively by drainage of the thorax and maintenance of nutrition. When the daily output of chyle was more than 1000 ml, with no decrease after 4 to 5 days, re-operation was performed with ligation of the thoracic duct (Wang et al., 1985). The incidence of chylothorax following supradiaphragmatic ligation of the main thoracic duct was 0.7%, compared with 1.6% in those who did not undergo ligation of the thoracic duct (Li et al., 2001). Since 1990, the thoracic duct has therefore been ligated routinely as part of oesophagectomy for cancer.

Although uncommon, surgical intervention was usually necessary for postoperative bleeding and diaphragmatic herniation. From 1952 to 1993, postoperative bleeding that required surgical intervention occurred in 22 (0.3 %) of 8,417 patients who underwent oesophagectomy for cancer, resulting in three deaths (Zhang et al., 1993). Postoperative bleeding originated from the intercostal artery, the cut end of the rib, damaged spleen, short gastric vessels, the infradiaphragmatic artery, and vessels in the great and lesser omentum (Zhang et al., 1993). The indications for reoperation for bleeding were overt blood loss exceeding 200 ml/h for at least 3 hours, with symptoms and signs of shock; a haemoglobin concentration in drained blood of more than 5g/l; or, if neither of the above criteria was met, the presence of clinical haemorrhagic shock with radiographic evidence of increasing pleural fluid. Oesophagoarterial fistula was a rare complication; between 1952 and 1979 the incidence was 0.4 % (ten of 2,748) and all ten patients died (Zhang et al., 1993). Ideally the anastomotic site should not be close to large vessels, especially the aorta. If this was unavoidable, a pedicled pleural flap or fat pad could be used to separate the anastomotic site from the aorta. The incidence of oesophagoarterial fistula decreased significantly in recent years, probably as a result of this measure. Diaphragmatic herniation after oesophagectomy was also rare. Between 1952 and 1981, 16 (0.4 %) of 4,148 patients developed significant diaphragmatic herniation,

usually involving the transverse colon and greater omentum (Ping et al., 1983). Fourteen of the 16 patients underwent repair of the hernia via a thoracic approach and two via the abdomen (Ping et al., 1983).

The postoperative mortality rate (death within 30 days) decreased, especially in the latter part of the series (Table 3-2). Up to December 1978, 190 (4.4 %) of 4,310 patients who underwent surgery died within 30 days, compared with 1.1% in the last decade. Between September 1952 and December 1978, the main causes of postoperative death were anastomotic leakage, followed by traumatic shock, pulmonary complications, empyema, cardiovascular complications and chylothorax in descending order (Zhang et al., 1982). There were changes in the causes of postoperative death from January 1966 to May 1994 (Ping et al., 1995). Anastomotic leakage remained the leading cause of death, followed by pulmonary complications, cardiovascular complications, empyema, large-artery leakage, chylothorax and pseudomembranous colitis. Traumatic shock was no longer a major cause of death, probably due to improvements in surgical technique and postoperative care

3.7. Long-term outcome

From the establishment of the department in 1952 to the end of the 1980s, the 5-year survival rate for SCC of the oesophagus and adenocarcinoma of the gastric cardia improved gradually (Department, 1978; Zhang et al., 1982; Zhang et al., 1984; Zhang et al., 1989; Adachi et al., 1996). In the 1990s, however, the 5-year survival rate fell slightly (Ping, 2002). This was probably because resections of more advanced stage cancers were attempted, as a result of improvements in surgical techniques and perioperative care. The 5-year survival rate for adenocarcinoma of the gastric cardia remained lower than that for SCC of the oesophagus.

The length of cancer, the extent of lymph node metastasis, and the depth of cancer

invasion have all been significantly associated with the 5-year survival (Zhang et al., 1982). For the 4,310 patients operated on between 1952 and 1978, the 5-year survival rates for patients with oesophageal cancers of less than 3, 3-5 and larger than 5 cm in length were 43.9%, 28.4% and 24.4%, respectively ($P<0.05$) (Zhang et al., 1982). The five-year survival rate for patients who had positive lymph nodes was 10%, compared with 39.3% for those who had negative nodes ($P<0.01$). The corresponding figures for those with cancer of the gastric cardia were 8.3% and 26.8% ($P<0.05$). The 5-year survival rate for those with oesophageal cancer confined to adventitia was 42.3%, and that for patients who had invasion beyond adventitia was 29.5% ($P<0.01$). The respective values were 10.0% and 14.1% for patients with cancer of the cardia invading beyond or confined to serosa, respectively ($P>0.05$) (Zhang et al., 1982). For 141 patients with superficial cancers of the oesophagus or gastric cardia, the 5-year survival rates were 75.5% and 71.1%, respectively ($P>0.05$) (Liu et al., 1995). These results indicate that the prognosis for patients with early cancer of the oesophagus or gastric cardia is much better than that for patients with advanced cancers, emphasizing the need for early diagnosis to improve long-term survival.

Factors affecting the survival of patients undergoing oesophagectomy for cancer between 1985 and 1989 were analyzed by means of the Cox's proportional hazard model (Zhang et al., 1999). Multivariate analysis showed that the major significant prognostic factors influencing survival were lymph node metastasis, TNM stage, depth of invasion, and histological type. Sex, age, site of tumour, length of stump and preoperative radiation therapy were not associated with survival.

To determine the effect of the extent of resection on long-term survival, the outcome for 1,164 patients with thoracic oesophageal cancer treated in Hebei was compared to that for 140 patients treated at Shinshu University, Japan. For the Hebei patients, subtotal thoracic oesophagectomy with paraesophageal and cardiac lymph node dissection was routinely performed by left thoracotomy. For the Shinshu patients,

total thoracic oesophagectomy with two- or three-field lymph node dissection, which was more extensive than that in Hebei, was usually performed via right thoracotomy, upper midline laparotomy, and cervical incision. The Hebei patients with pT2 or pT3 tumour survived significantly longer than the Shinshu patients ($P<0.01$), but there was no significant difference between the two groups when the tumours were classified by pTNM stage (Adachi et al., 1996).

3.8. Adjuvant therapy

A randomized comparative study of pre-operative radiotherapy and surgery *versus* surgery alone was undertaken in 200 patients with oesophageal cancer between 1984 and 1985. One hundred patients received 40 Gy radiotherapy to the mediastinum in 20 fractions over 25 days, followed by surgery 2 to 4 weeks after irradiation. The other 100 underwent surgery alone. The resectability rate was the same in both groups (90% vs 90% $P>0.05$), and there were no differences in the 5-year survival (34% vs 30% $P>0.05$) (Zhang et al., 1998). A retrospective study of postoperative radiotherapy for oesophageal cancer was carried out in 1,064 patients, including 82 patients who received postoperative radiotherapy and 982 who underwent surgery only. Radiotherapy was started 2 to 6 weeks after surgery, at a dose of 60 Gy in 30 fractions over 40 days to the mediastinal region, and 50 Gy in 25 fractions over 30 days in each supraclavicular region (Liu et al., 1996). The 5-year survival rate was higher after radiotherapy and resection (42%) than after surgery alone in stage IIa cancers (26%) ($P<0.01$), but there were no significant differences in survival amongst patients with stage III or IV diseases (Liu et al., 1996).

3.9. Summary

Southern Hebei Province has a high incidence of oesophageal cancer. In the past 30 years, there has been a concerted effort by the Chinese government to prevent the disease. While the incidence of oesophageal cancer has fallen, the treatment of the disease remains challenging because of a high recurrence rate and disappointing

long-term survival. More than 17,000 patients have had an oesophagectomy for cancer at the Fourth Hospital, Hebei Medical University since 1952. There has been a gradual reduction in postoperative mortality and morbidity over this time, although the long-term survival remains disappointing. The greatest impact on long-term survival is likely to stem from diagnosis of the disease at the early stage. More encouraging results may be possible with careful selection of patients for operation and better use of radiation and other adjuvant therapy.

Table 3-1. The resectability rate for the patients with carcinoma of the oesophagus or gastric cardia treated in the Department of Thoracic Surgery, Fourth Hospital, Hebei Medical University, China.

The year of report	Years of operation	No. of patients	Oesophageal cancer			Cardiac cancer			Overall resection rate (%)
			n	No. Resected	Resectability rate (%)	n	No. resected	Resectability rate (%)	
1978 (*)	1952-1976	3694	2225	1754	78.8	1469	1023	69.6	75.2
1982 (Zhang, et al)	1952-1978	4310	2568	2027	76.3	1742	1251	71.8	77.1
1984 (Zhang, et al)	1952-1982	5587	3308	2733	82.6	2279	1680	73.7	79.0
1989 (Zhang, et al)	1952-1988	9601	---	---	87.3	---	---	78.2	83.7
1996 (Adachi, et al)	1984-1989	1164	1164	1087	93.4	---	---	---	93.4
2002 (Ping, et al)	1990-2000	6894	4016	3856	96.0	2827	2727	94.8	95.5

* Department of Thoracic Surgery, Fourth Hospital, Hebei Medical University

Table 3-2. Major complications and deaths after oesophagectomy for cancer in the Department of Thoracic Surgery, Fourth Hospital, Hebei Medical University, China.

The year of report	Yrs of Operation	No. of Patients	No. of Resected	Anastomotic leakage (%)	Pulmonary complication (%)	Empyema (%)	Chylothorax (%)	Cardiovascular Complication (%)	Death (%)
1978 (*)	1952-1976	3694	2777	5	3.2	4.5	0.8	1.3	4.6
1982 (Zhang, et al)	1952-1978	4310	3321	4.8	2.9	4.2	0.8	1.2	4.4
1984 (Zhang, et al)	1952-1982	5587	4413	4.4	2.6	3.8	1	1.1	4.5
1989 (Zhang et al)	1952-1988	9601	8033	3.4	---	---	---	---	---
1996 (Adachi et al)	1984-1989	1164	1087	3.4	1.5	---	0.7	---	1.8
2002 (Ping, et al)	1990-2000	6894	6583	---	---	---	---	---	1.1

* Department of Thoracic Surgery, Fourth Hospital, Hebei Medical University

Table 3-3. Five-year survival rate for the patients with cancer of the oesophagus or gastric cardia treated in the Department of Thoracic Surgery, Fourth Hospital, Hebei Medical University, China

Year of report	Yrs of operation	No. of patients	Oesophageal cancer		Cardiac cancer	
			Number	5-yr survival %	Number	5-yr survival %
1978 (*)	1952-1976	3694	2225	21.2	1023	15.2
1982 (Zhang, et al)	1952-1978	4310	2568	23.5	1742	15.4
1984 (Zhang, et al)	1952-1982	5587	3308	24.9	2279	19.0
1989 (Zhang et al)	1952-1988	9601	---	29.2	---	17.8
1996 (Adachi et al)	1984-1989	1164	1164	43.5	---	---
2002 (Ping, et al)	1990-2000	6894	4016	23.9	2878	17.6

* Department of Thoracic Surgery, Fourth Hospital, Hebei Medical University

Chapter 4. The expression of COX-2 in squamous cell carcinoma of the oesophagus

4.1. Introduction

In epidemiological studies, as reviewed in Chapter 1, aspirin and non-steroidal anti-inflammatory drugs (NSAIDs) have been reported to have a chemopreventative effect in a variety of cancers, including oesophageal cancer. The ability of NSAIDs to suppress inflammation is thought to rest primarily on their ability to inhibit the cyclooxygenase (COX)-2 enzyme. Increased expression of COX-2 protein, and its correlation with tumour stage and mortality has been reported in SCC of the oesophagus and many other cancers such as stomach, breast, ovarian, bladder and lung. It has also been demonstrated that COX-2 is up-regulated in the progression from normal squamous epithelium through dysplasia to SCC of the oesophagus. These findings suggest that COX-2 expression may be related to the development of oesophageal cancer, and thus could be a useful marker for prognosis in this disease, as well as a target for therapy.

In this study immunohistochemistry was used to determine the distribution of COX-2, and flow cytometry (FCM) to quantitate its expression in tissues resected for oesophageal SCC. Its significance as a prognostic indicator was also determined. The present study was approved by the Ethics Committee, the Fourth Hospital, Hebei Medical University, China. Informed consent was obtained from all patients.

4.2. Results

4.2.1. Patients

The study group consisted of 90 men and 48 women, with a mean age of 53 years, who underwent oesophagectomy for SCC in the Department of Thoracic Surgery, Fourth Hospital, Hebei Medical University, China from October 1984 to May 1985. None of these patients received any pre-operative treatment such as chemotherapy or radiotherapy. Paraffin blocks of specimens resected from these patients were retrieved for immunohistochemical and flow cytometric analyses, and medical records

were reviewed for clinical data. Survival time was calculated from the day of surgery to death or until November 20, 2000. Survival analysis was carried out with a minimum follow-up of 180 months and a maximum of 199 months. The tumours were classified as T2N0M0 (23 patients), T2N1M0 (7), T3N0M0 (63) and T3N1M0 (45). One hundred and one cancers were in the middle third of the oesophagus, 36 were in the lower third and 1 was in the upper third. In addition, 21 cancer-free margins of the oesophagus and 23 obtainable metastatic lymph nodes were also immunostained for COX-2 to compare to the primary tumours.

4.2.2. Immunohistochemistry

Sections were stained and assessed as described in Section 2.1. All sections of colon cancers which were used as positive controls were strongly stained for COX-2, but no staining was observed in the negative controls in which the primary antibody was omitted. In the 21 cancer free oesophageal margins of the resected specimens, staining for COX-2 was found predominantly in the cells of the basal layer of the histologically normal squamous epithelium (62% of cases), in smooth muscle cells (71%), in vascular endothelial cells (81%), and mononuclear inflammatory cells (52%).

COX-2 was detected in 77 (56%) of the oesophageal SCC. Of the 77 COX-2 positive cancers, cells with cytoplasmic staining were patchily distributed through the section in 44 (57%) cases, or were distributed throughout the whole section in 33 (43%) cases. COX-2 staining, confined to the cytoplasm, was detected in smooth muscle cells in 101 (73%) cases, in mononuclear inflammatory cells in 51 (37%) cases, in vascular endothelial cells in 43 (31%) cases, and in submucosal glands in each of the 15 sections in which the glands were seen. Most metastatic cancer tissue in lymph nodes showed the same extent of COX-2 staining as seen in the corresponding primary tumours (Figure 4-1).

The grades of staining for COX-2 are shown in Figure 4-2. They were significantly higher in both primary and metastatic cancers than in normal squamous epithelia ($P = 0.002$ and $P < 0.0001$ respectively). A significant increase in the grade of staining for COX-2 was observed in the metastatic cancer in lymph nodes compared to the

matched primary cancer (P = 0.005).

The results in Table 4-1 show the relationship between COX-2 expression and clinicopathological parameters. The extent of staining for COX-2 correlated positively with the finding of lymph node metastases (P = 0.03). The mean survival time of all patients was 84.3 months. The mean survival time of the patients (N = 100) with 10% or fewer COX-2 positive cancer cells was 99.4 months, significantly longer than the 56.7 months for patients (N = 38) with more than 10% positive cells (t = 2.54, P = 0.01). Furthermore, the 5-year survival rate in patients with less than 10% COX-2 positive cells was 47.5%, compared to 23.2% in patients with more than 10% COX-2 positive cells (Figure 4-3) (log-rank x^2 = 8.47, P = 0.0036).

Table 4-1. Relationship between grades of staining for COX-2 in the primary oesophageal SCC and clinicopathological features.

	Kendall statistic	P-value
Sex	-0.150	0.079
Age	0.001	0.845
Length of tumour	-0.140	0.057
Depth of tumour invasion	0.127	0.129
Lymph node involvement	0.186	0.030
Vascular invasion	0.039	0.650
Differentiation	-0.070	0.403

One possible explanation for the relationship between survival and COX-2 expression could be that COX-2 expression is a surrogate marker for tumour stage, with tumour stage being related to survival. Kaplan-Meier survival analysis was performed on 3 subgroups of patients categorised on the basis of tumour stage. There was no difference in survival for patients with 10% or fewer COX-2 positive cancer cells compared to those with more than 10% in the T2N0M0 (N = 18 *vs* 5; P = 0.85) or the T3N1M0 (N = 21 *vs* 24; P = 0.068) subgroups, although patients with < 10 % of positive cell in their tumour survived significantly longer in the T3N0M0 subgroup (N = 45 *vs* 8; P = 0.0083).

4.2.3. Flow cytometry

4.2.3.1. Morphological observation and flow cytometry histogram analysis

Cell suspensions, prepared from archival formalin fixed, paraffin embedded tissue

were immunostained for COX-2 and examined as described in Section 2.2. The staining as visualised by fluorescence microscopy was predominantly localized to within the cytoplasm (Figure 4-4). Typical histograms from flow cytometry of these cells are shown in Figure 4-5. Figure 4-5A shows COX-2 expression in cells from normal oesophageal tissue, with a fluorescence index (FI) value of 0.83. Figure 4-5B shows increased expression of COX-2 in cells from a SCC of the oesophagus, with a FI value of 1.5. Figure 4-5C shows cells from metastatic cancer in a lymph node, with a FI value of 1.5. Overall, cells staining for COX-2 were present in 95.7% (132/138) of the primary oesophageal cancers.

4.2.3.2. Fluorescence index in primary cancers, metastatic lymph nodes and normal tissues of the oesophagus

The FI values of cells prepared from the primary tumour, the cancer positive lymph nodes, and the histologically normal resection margins were compared (Figure 4-6). Cells from both the primary cancer and metastatic cancer had significantly higher FI values than from the resection margins (for both, $P < 0.001$). However, there was no significant difference in FI between cells from the primary tumours and the metastatic lymph nodes ($P = 0.651$).

4.2.3.3. The extent of COX-2 expression and clinicopathological features

Results in Table 4-2 show the correlation between the expression of COX-2 in the primary tumour as measured by FCM (as described in Section 2.2) and clinicopathological features. Expression of COX-2 was significantly associated with differentiation of the cancer. The rate was higher in well and moderately differentiated cancers than in poorly differentiated lesions ($P = 0.02$).

The mean survival time was 82.9 months for the entire group. The survival rates were also calculated with the patients stratified into to a high COX-2 expression group ($FI > 1.98$) or a low COX-2 expression group ($FI < 1.98$). There was no statistical difference in the 5-year survival rate in the low COX-2 expression group (41.2%) compared to the high COX-2 expression group (36.4%) (Figure 4-7; $P = 0.4417$).

4.3. Conclusion

The results in this chapter show that COX-2 is highly expressed in oesophageal SCC and lymph node metastases compared to the the histologically normal tissue at the proximal oesophageal resection margin. Two methods were used for quantitating the COX-2 expression from the same archived formalin fixed paraffin embedded tissues. Increased COX-2 expression measured by immunohistochemistry was associated with lymph node metastases and shorter survival time. Flow cytometry was more sensitive than immunohistochemistry, with COX-2 being detected in more of the tumours. There was no association between increased expression of COX-2 as measured by the fluorescence intensity from flow cytometry and survival. No attempt was made to integrate the flourescence intensity and the percentage of tumour cells expresing COX-2, which might have given a result similar to that from the immunohistochemistry.

Table 4-2. The expression of COX-2 in oesophageal cancers as detected by flow cytometry. and its relationship with clinicopathological features.

	N	*Tumours expressing COX-2 (% of total tumours)*	*P*
Sex			
Male	90	95.6% (87/90)	0.97
Female	48	95.7% (45/48)	
Age			
≤60	122	86.9% (106/122)	0.36
>60	16	100% (16/16)	
Site of tumour			
Upper/middle	102	96.1% (99/102)	0.88
Lower	35	88.6% (31/36)	
Length of tumour			
≤5cm	98	96.9% (95/98)	0.25
>5 cm	40	92.5% (37/40)	
Differentiation			
Well+Moderately	113	97.3% (110/113)	0.02
Poorly	25	84.0% (21/25)	
Depth of invasion			
T2	31	90.3% (28/31)	0.61
T3	107	86.9% (93/107)	
Nodes			
N0	88	95.5% (84/88)	0.88
N1	50	96.0% (48/50)	
UICC Stage			
II	93	93.5% (87/93)	0.18
III	45	100% (45/45)	

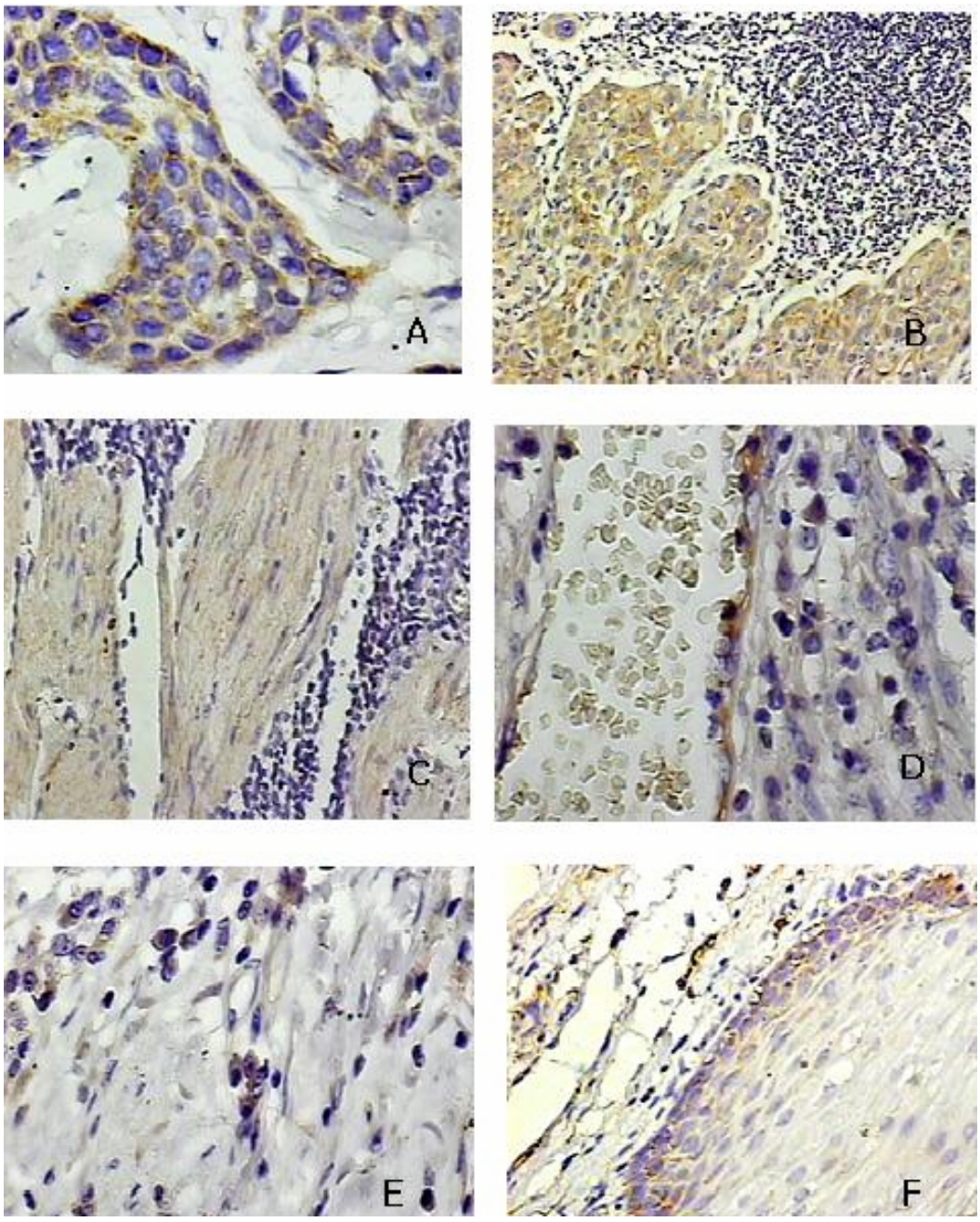

Figure 4-1. Immunohistochemical staining for COX-2 (brown colouration) in a primary SCC of the oesophagus (A, magnification 400×) and its corresponding metastatic lymph node (B, magnification 100×). Smooth muscle cells (C, magnification 100×), vascular endothelial cells (D, magnification 200×) and mononuclear inflammatory cells (E, magnification 200×) were also stained in a cancer affected oesophagus. COX-2 positive cells were predominantly located in basal layer cells of normal squamous epithelium (F, magnification 100×).

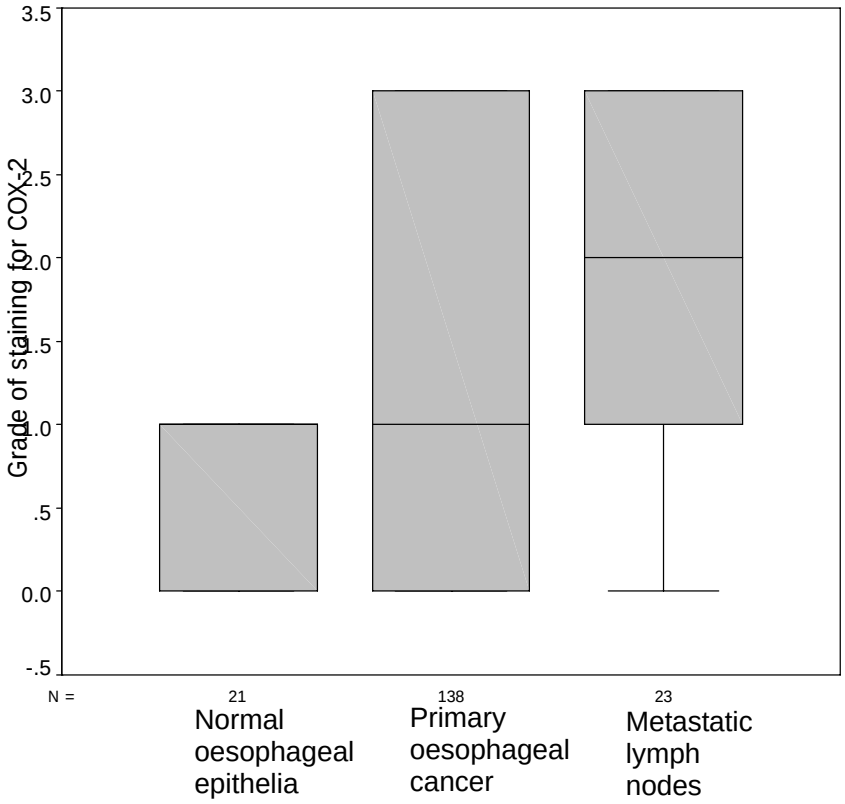

Figure 4-2. Comparison of the grades of immunostaining for COX-2 in SCC of the oesophagus, metastatic lymph nodes and cancer-free oesophageal stumps. Horizontal lines represent median values; boxes denote the interquartile range. A significant increase of the extent of staining for COX-2 was determined in primary oesophageal cancers (P = 0.002) and in metastatic lymph nodes (P < 0.0001) versus normal oesophageal tissues. The grades of staining in metastatic lymph nodes were higher than in primary oesophageal cancers (P = 0.005).

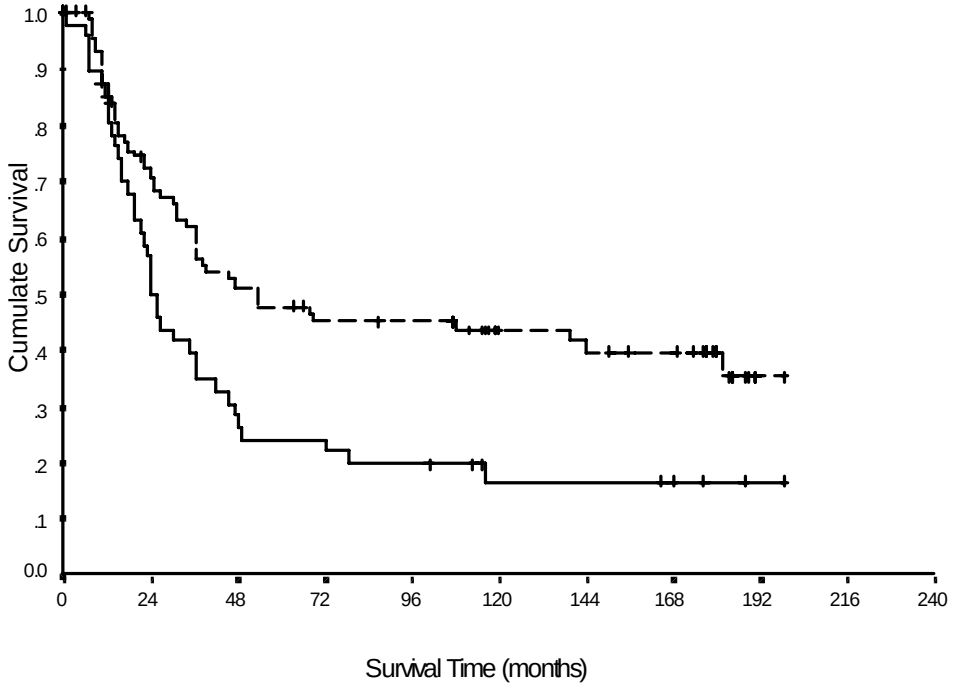

Figure 4-3. Kaplan-Meier survival curves stratified according to COX-2 grading by immunohistochemistry in SCC of the oesophagus. Patients with COX-2 expression of Grades 0 - 1 (broken line) (N = 100) had a significantly longer survival than those with Grades 2 - 3 (solid line) (N = 38) (P = 0.0036).

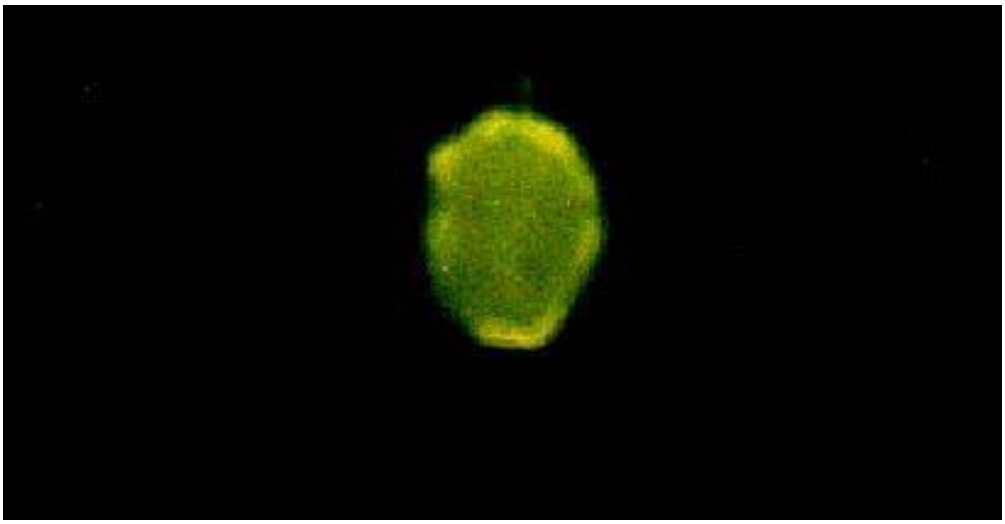

Figure 4-4. Fluorescence staining for COX-2 (yellow colouration) in an oesophageal SCC cell prepared from formalin fixed paraffin embedded tissue. The COX-2 protein is located predominantly in the cytoplasm (fluorescence microscopy ×400).

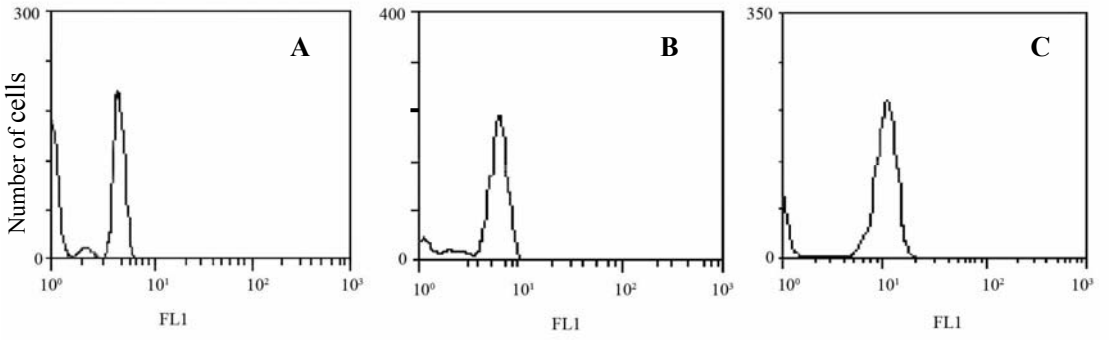

Figure 4-5. Flow cytometric histograms of COX-2 from formalin fixed paraffin embedded normal oesophagus, SCC of the oesophagus and metastatic lymph node tissues. (A) Normal tissue from the oesophageal resection margin (FI value is 0.83）. (B) Primary SCC of the oesophagus (FI value is 1.5）. (C) Metastatic lymph node (FI value is 1.5).

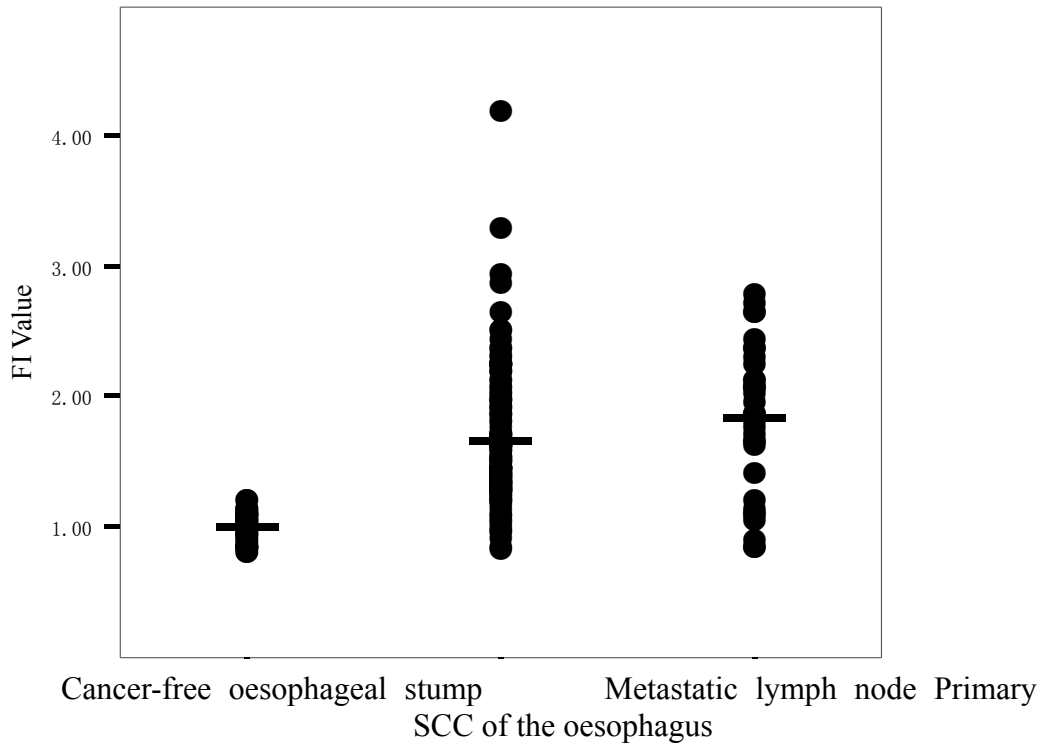

Figure 4-6. Comparison of fluorescence index measured by flow cytometry in cells from formalin fixed paraffin embedded cancer-free oesophageal resection margin, primary SCC of the oesophagus and metastatic lymph node tissues. Horizontal lines represent median values. Compared to the cancer-free oesophageal margin, there was a significant increase in primary tumour ($P < 0.001$) and in the metastatic lymph nodes ($P < 0.0001$). There was no significant difference between the primary tumours and the metastatic lymph nodes ($P = 0.651$).

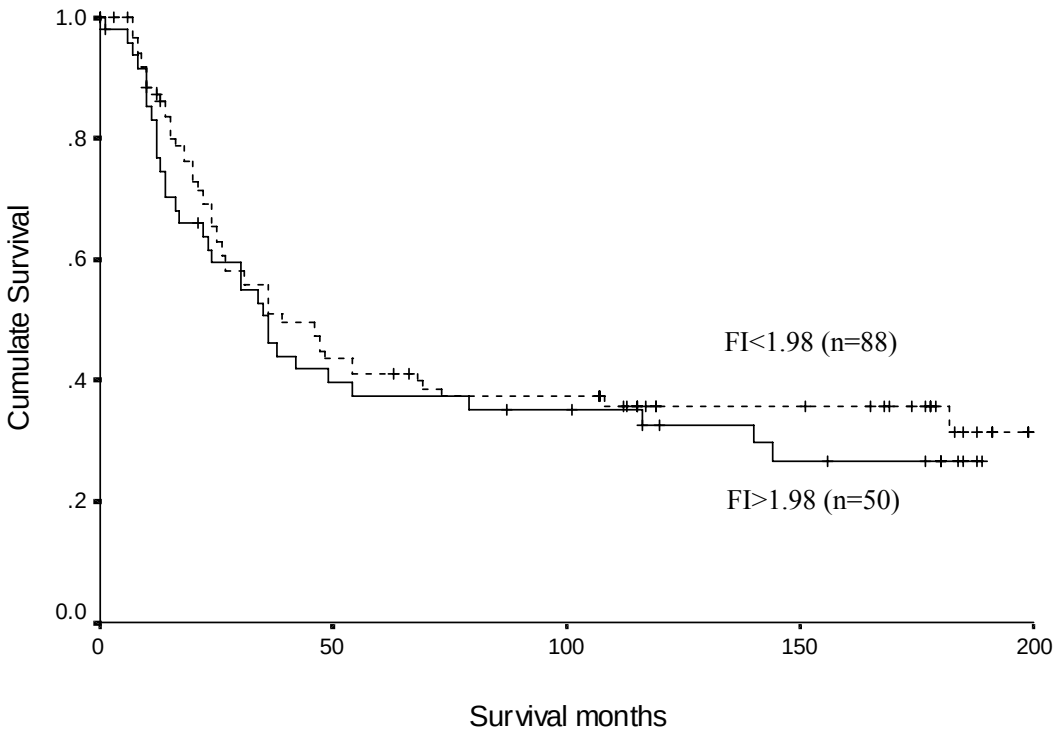

Figure 4-7. Kaplan-Meier curves stratified according to fluorescence index values measured by flow cytometry in cells from primary tumours of SCC of the oesophagus. The five year survival rate was 41.2% in the low COX-2 expression group (FI values < 1.98), and 36.4% in the high COX-2 expression group (FI values>1.98) (P = 0.4417).

Chapter 5. The effects of aspirin and NS-398 on an oesophageal cancer cell lines

5.1. Introduction

Epidemiological and experimental studies reviewed in Chapter 1 have demonstrated the potential of aspirin and non-steroidal anti-inflammatory drugs (NSAIDs) for the chemoprevention of SCC. The effects of NSAIDs are thought to be mediated mainly through the inhibition of the cyclooxygenases (COXs), which catalyze the rate-limiting step in the formation of prostaglandins from arachidonic acid. There are two isoforms of cyclooxygenase, COX-1 and COX-2. COX-1, constitutively expressed at near constant levels and activity in many tissues, has a role in a number of physiological functions. COX-2, normally undetectable in most tissues, is an inducible or early-response gene, as well as frequently being over-expressed in tumours, including oesophageal SCC and adenocarcinoma (Wilson, et al., 1998; Zimmermann, et al., 1999; France, et al., 2004). The increased concentrations of prostaglandins synthesised by the COX-2 can drive tumour growth by promoting angiogenesis, inhibiting apoptosis, stimulating cell proliferation and motility, and modulating inflammatory and immune responses (Xu, 2002; Zha, et al., 2004).

NSAIDs can inhibit COX, but a number of studies have suggested that they can also affect cells by other mechanisms. Aspirin has been reported to inhibit the activation of the NF-KappaB pathway. These transcription factors are important regulators of immune and inflammatory responses, and are involved in preventing apoptosis in response to nuclear damage or cytotoxic cytokines. The purpose of this study was to measure the effect of aspirin, and of a selective COX-2 inhibitor NS398, on these pathways in the human oesophageal SCC cell line TE-13.

5.2. Results

5.2.1. Effects of aspirin on an oesophageal cancer cell line

5.2.1.1. Effect of aspirin on proliferation and apoptosis

The human oesophageal SCC cell line TE-13 was cultured as described in Chapter 2.3,

and proliferation was measured as described in Chapter 2.4. The effect of aspirin on the proliferation of TE-13 was measured after the culturing of the cells with 1, 4 or 8 mmol/L added to the medium. Compared to cells grown in its absence, aspirin resulted in a significant reduction in proliferation of 44.8 ± 19.1%, 50.3 ± 10.9% and 61.9 ± 19.3% respectively ($P < 0.05$ for each). The percentage of apoptotic cells, measured as described in Chapter 2.5, after 24 hours of culture in the same concentrations of aspirin was 13.8 ± 2.3%, 13.9 ± 2.9% and 14.6 ± 1.9% respectively, each significantly greater than the 4.6 ± 3.2% in the cells cultured in the absence of aspirin ($P < 0.05$ for each).

A sample of the cultured cells was examined by electron microscopy, as described in Chapter 2.6. The cells cultured with aspirin showed an abnormal ratio of cytoplasm to nucleus, nuclear pyknosis and cytoplasmic condensation (Figure 5-1A). However, only chromatin margination was found in untreated cells (Figure 5-1B).

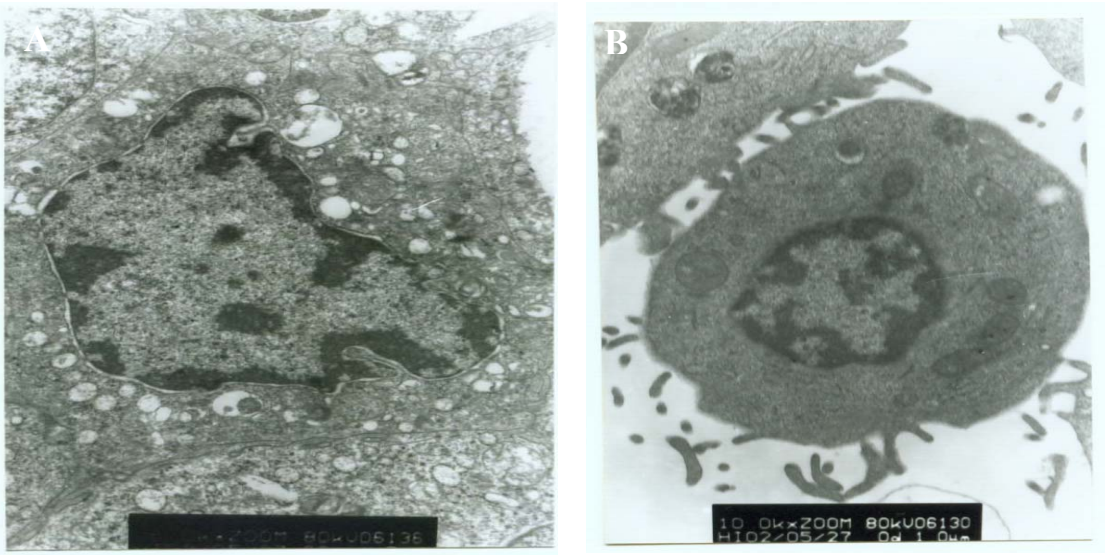

Figure 5-1. Representative electron micrographs of cultured TE-13 cells. (A) A TE-13 cell grown in the absence of aspirin. The nucleus is dysmorphic with a prominent nucleolus, and heterochromatin margination is present (original magnification 6,000×) (B) A TE-13 cell grown in the presence of aspirin. The ratio of the nucleus to the cytoplasm is abnormal. Nuclear pyknosis and cytoplasmic condensation are present. (original magnification 10,000×).

5.2.1.2. Effect of aspirin on PGF1α production

The concentration of $PGF_{1\alpha}$ (measured as in Chapter 2.7) in the supernatant of TE-13 cells incubated for 48 hours in the absence of aspirin was 35.9 ± 9.8 pg/ml. In cells incubated for the same period with aspirin added to the medium at a concentration of 1, 4, or 8 mmol/L, $PGF_{1\alpha}$ production was significantly reduced to 15.8 ± 8.1, 23.7 ± 6.6 and 16.7 ± 4.2 pg/ml respectively ($P < 0.05$ for each).

5.2.1.3. Effect of aspirin on COX-2 mRNA expression

The level of COX-2 mRNA in the cells was measured semiquantitatively by end-point PCR (as in Chapter 2.8). The results in Figure 5-2 show that cells cultured with 1, 4, or 8 mmol/L of aspirin for 24 hours had 19.1%, 31.1%, and 43.0% less transcript compared to cells without aspirin ($P < 0.05$).

5.2.1.4. Effect of aspirin on COX-2 protein expression

The results in Figure 5-3 show the effect of aspirin on the intracellular concentration of COX-2 as measured by Western blotting (Chapter 2.9). Incubation of the cells for 24 hours in medium to which either 1, 4 or 8 mmol/L of aspirin had been added each resulted in a significant decrease of 13.8%, 18.8% and 20.8% respectively in COX-2 protein expression ($P < 0.05$ for each). The results in Figure 5-4 show that there was a consistent reduction over time in the COX-2 protein concentration in cells harvested between 30 minutes to 48 hours following the addition of 4 mmol/L aspirin to the culture medium.

5.2.1.5. Effect of aspirin on NF-KappaB activation

The presence of NF-KappaB in the nuclear extract was determined by its binding to its consensus sequence in an electrophoretic mobility shift assay (EMSA) (Chapter 2.10). Binding specificity in the reaction was confirmed with the use of homologous (NF-KappaB) and nonhomologous (AP-2) oligonucleotides as competitors (Figure 5-5c). Positive control was provided in the reagent kit. The results in Figure 5-5A show that NF-KappaB was present in the control cells. Cells incubated for 24 hours with 1, 4 or 8 mmol/L of aspirin each showed a marked reduction in the amount of NF-KappaB. To determine the time course of this reduction, cells were incubated with 4 mmol/L of aspirin, and harvested at various time points up to 12 hours thereafter. The results in Figure 5-5B show that the reduction was induced within 30 minutes of the addition of the aspirin.

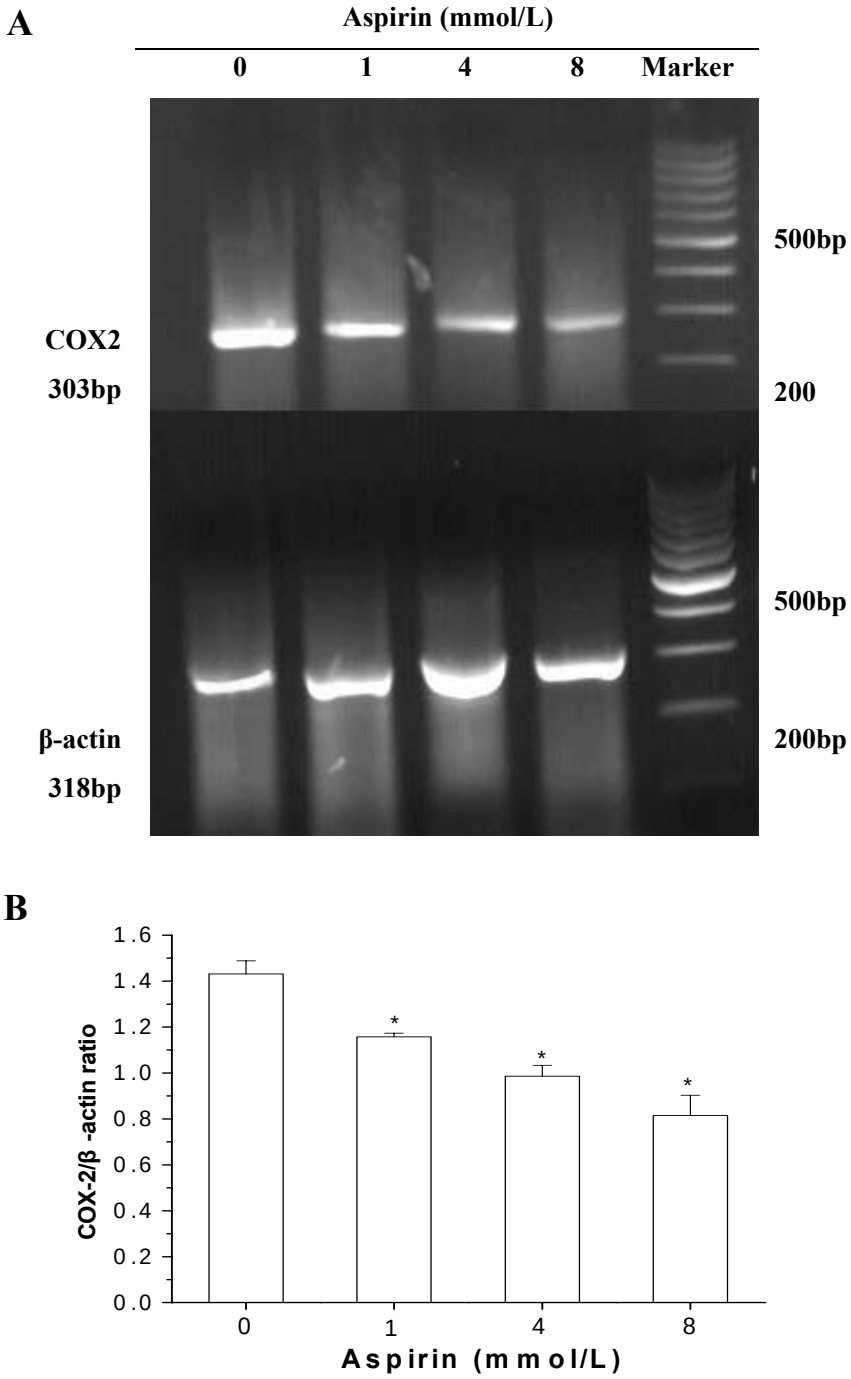

*Figure 5-2. The effect of the concentration of aspirin on the inhibition of COX-2 mRNA expression measured by RT-PCR in TE-13 cells. (A) A representative gel of COX-2 mRNA when aspirin was added at different concentrations. (B) COX-2 expression relative to expression of beta-actin as quantitated by Gel-Pro analyzer software (mean ± SEM of three independent experiments; * $P < 0.05$ compared to 0 mmol/L).*

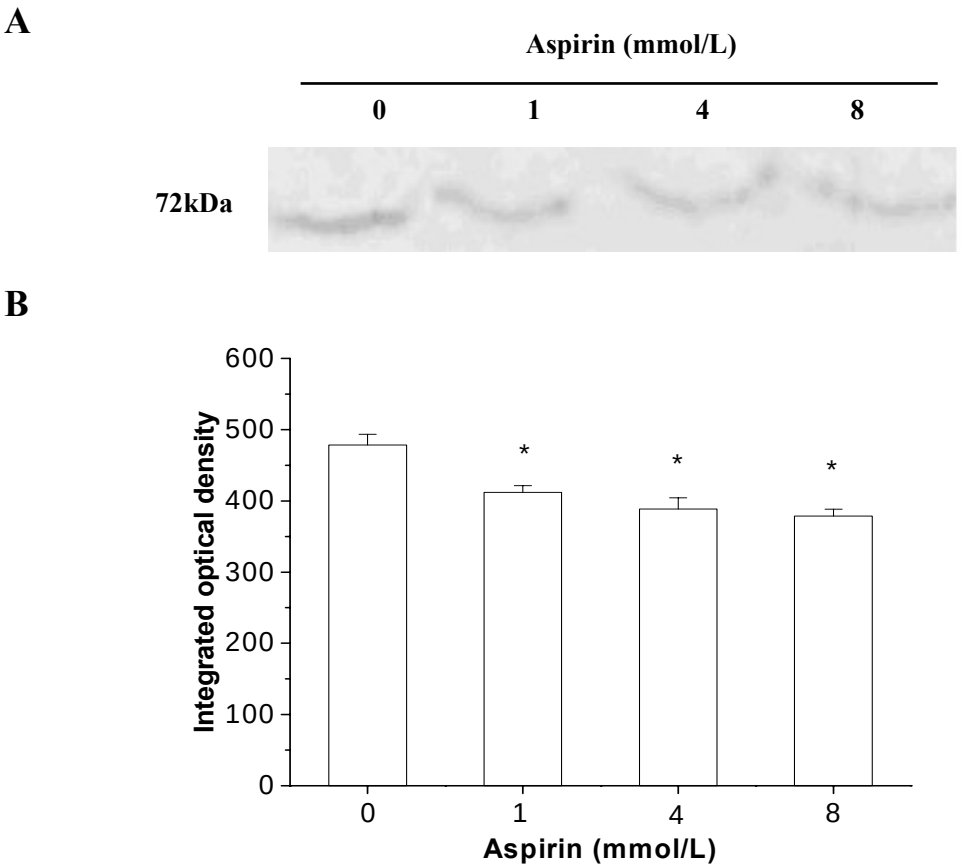

*Figure 5-3. The effect of the concentration of aspirin on the inhibition of COX-2 protein expression measured by Western blotting in TE-13 cells. (A) A representative gel of COX-2 protein concentration when aspirin was added to the cultures at the concentrations indicated. (B) COX-2 expression quantitated by Gel-Pro analyzer software (mean ± SEM of three independent experiments; * $P < 0.05$ compared to 0 mmol/L).*

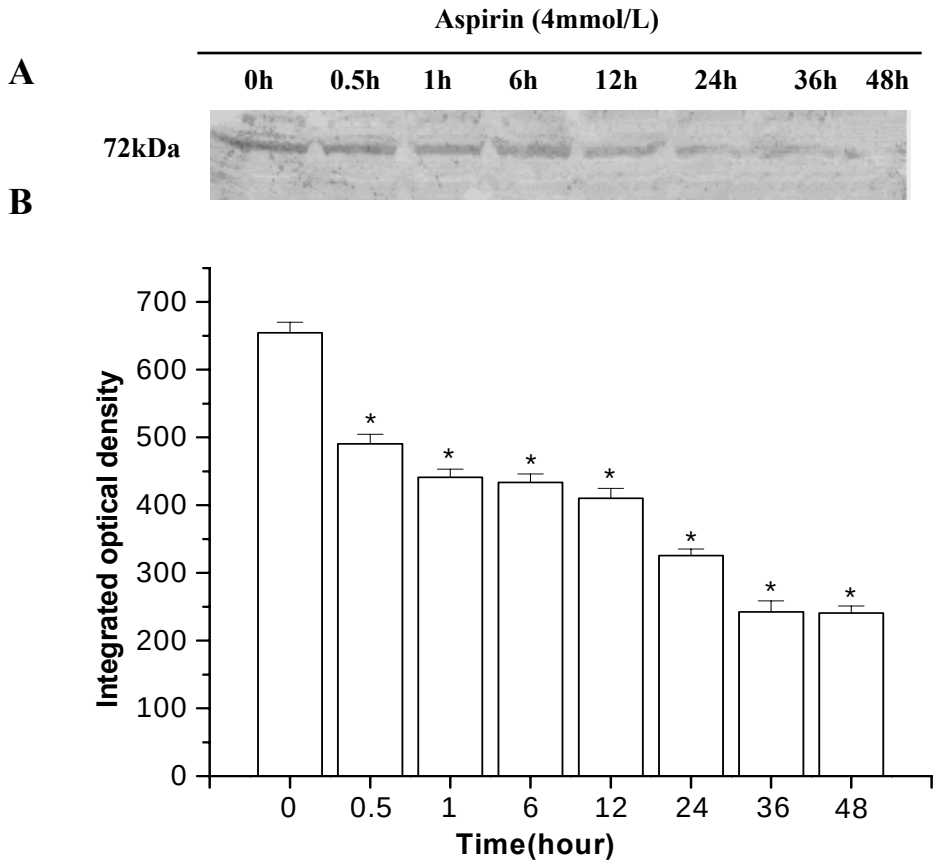

*Figure 5-4. The effect of time on the inhibition by aspirin of COX-2 protein expression measured by Western blotting in TE-13 cells. (A) A representative gel of COX-2 protein concentration when aspirin was added to the cultures for the times indicated. (B) COX-2 expression quantitated by Gel-Pro analyzer software (mean ± SEM of three independent experiments; * $P < 0.05$ compared to 0 hours).*

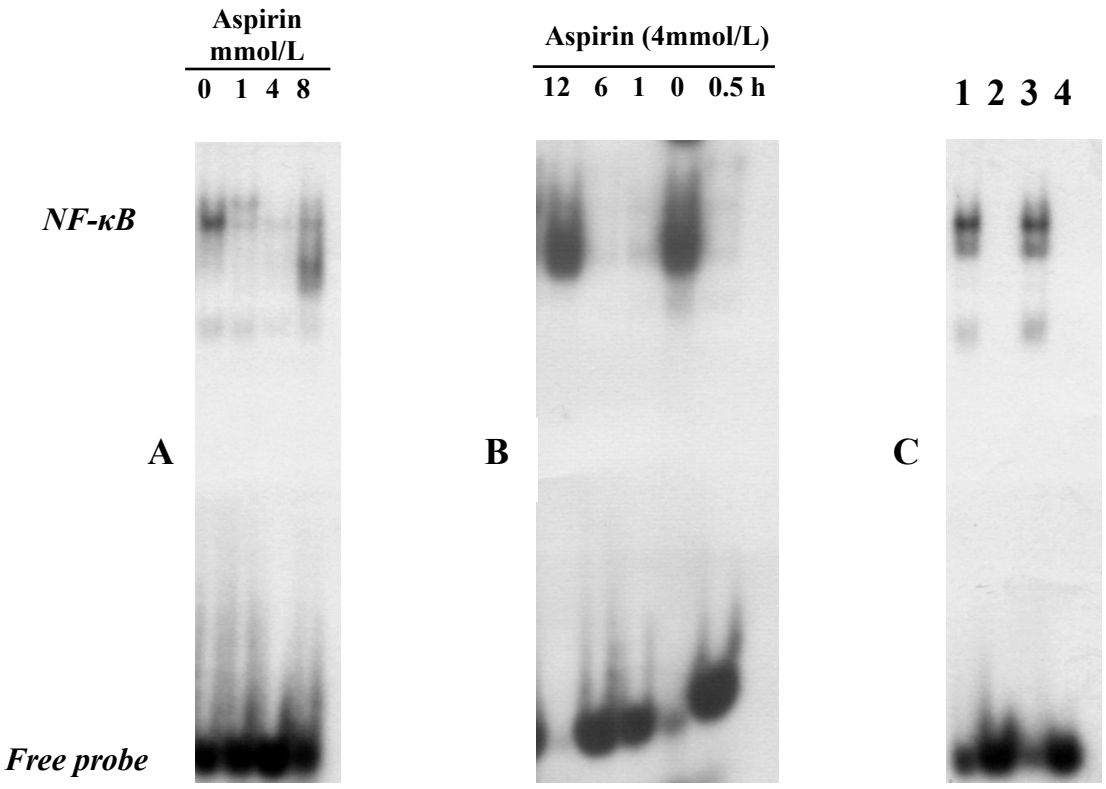

Figure 5-5. The effects of aspirin on the activity of NF-KappaB in TE-13 cells. (A) Aspirin inhibited NF-KappaB binding activity in a dose-dependent manner in TE-13 cells. (B) Aspirin inhibited NF-KappaB binding activity in a time-dependent manner in TE-13 cells. (C) The binding specificity was confirmed by using homologous (NF-KappaB) and nonhomologous (AP-2) oligonucleotides as competitors. Lane 1, positive control (Hela nuclear extract supplied by the manufacturer of the kit）; lane 2, negative control (nuclear extract omitted); lane 3, with the addition of unlabelled AP-2 oligonucleotide (noncompetitor); lane 4, with the addition of unlabelled NF-KappaB oligonucleotide (competitor).

5.2.1.6. Effect of aspirin on IKappaB protein level

The results in Figure 5-6 show the effect of aspirin on the intracellular concentration of IKappaB as measured by Western blotting. Incubation of the cells for 24 hours in medium to which 1, 4 or 8 mmol/L of aspirin had been added resulted in a significant increase of 45.3%, 89.1%, 102.3% respectively ($P < 0.05$ for each). The results in Figure 5-7 show there was a significant increase in the IKappaB protein concentration at all time points between 30 minutes and 48 hours following the addition of 4 mmol/L aspirin to the culture medium.

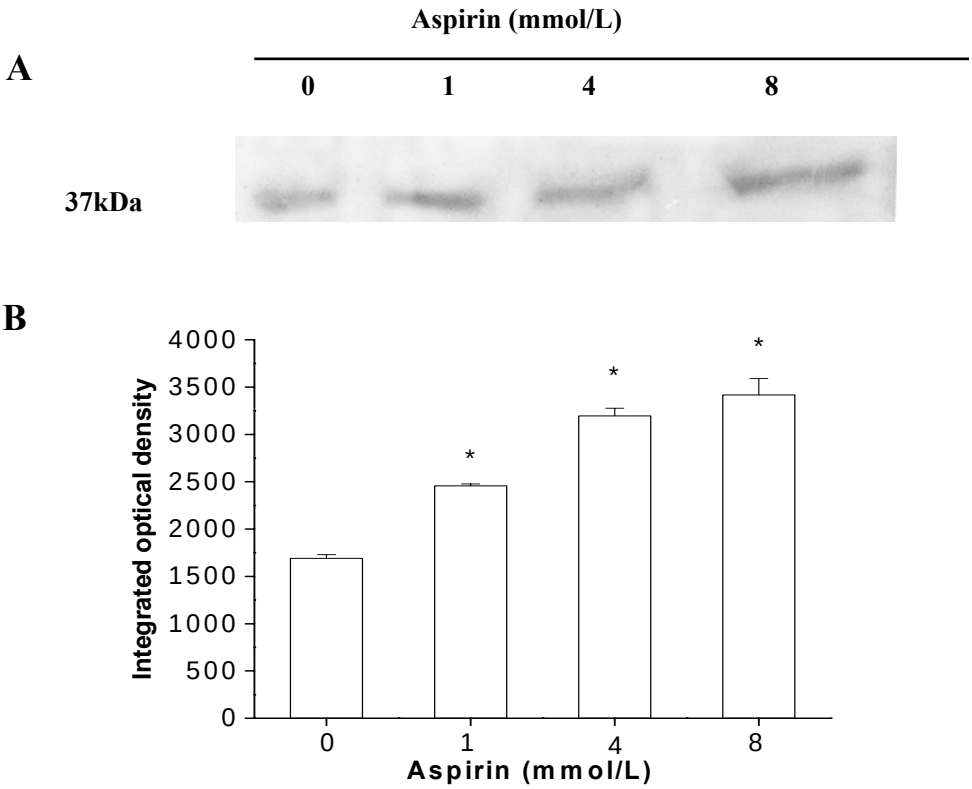

*Figure.5-6. The effect of the concentration of aspirin on the IKappaB protein level in the cytoplasm of TE-13 cells measured by Western blotting. (A) A representative gel of IKappaB protein concentration when aspirin was added to the cultures at the concentrations indicated. (B) IKappaB expression quantitated by Gel-Pro analyzer software (mean ± SEM of three independent experiments; * $P < 0.05$ compared to 0 mmol/L).*

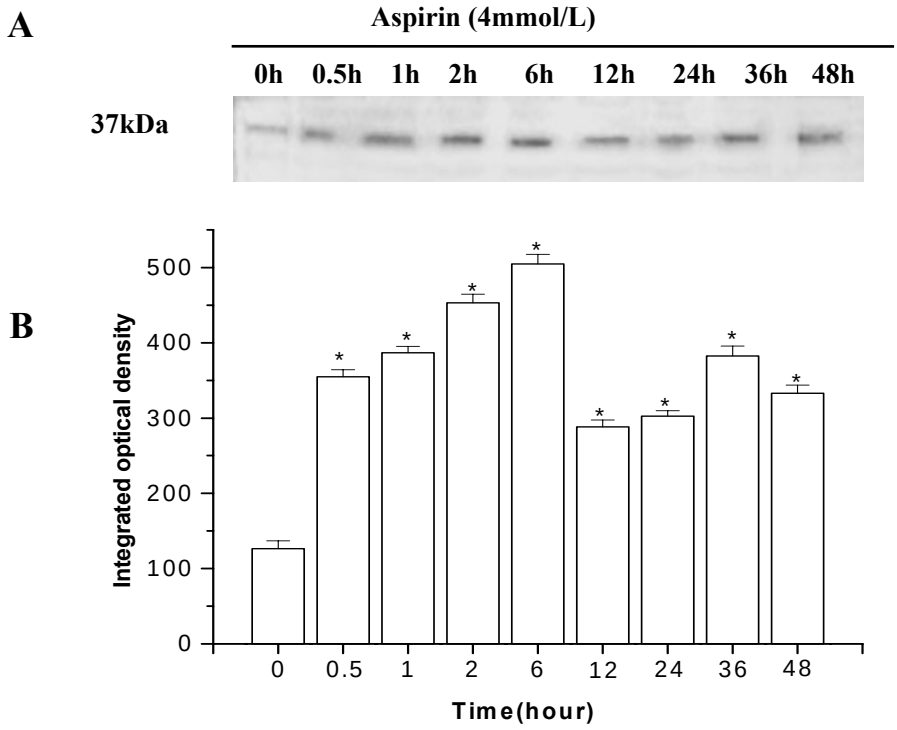

*Figure 5-7. The effect of time on the inhibition by aspirin of IKappaB protein level in the cytoplasm of TE-13 cells measured by Western blotting. (A) A representative gel of IKappaB protein concentration when aspirin was added to the cultures for the times indicated. (B) IKappaB expression quantitated by Gel-Pro analyzer software (mean ± SEM of three independent experiments; * P < 0.05 compared to 0 hours).*

5.2.2. Effects of NS-398 on an oesophageal cancer cell line

5.2.2.1. Effects of NS-398 on cell proliferation and apoptosis

The human oesophageal SCC cell line TE-13 was cultured as described in Chapter 2.3, and proliferation was measured as described in Chapter 2.4. The effect of NS-398 on proliferation of TE-13 was measured after culturing the cells for 72 hours with 0.001, 0.01, 1 or 100 μmol/L added to the medium. Compared to cells grown in its absence, NS-398 resulted in a significant reduction in proliferation of 50.2 ± 14.8%, 35.5 ± 18.2%, 15.8% ± 5.0%, 4.9 ± 3.5%, respectively (P<0.05 for each). The percentage of apoptotic cells, measured as described in Chapter 2.5, after 24 hours of culture in the same concentrations of NS398 was 5.4 ± 1.1%, 6.0 ± 0.8%, 11.3 ± 0.3%, 17.5 ± 2.4% respectively, each significantly greater than the 2.6 ± 1.6% in the cells cultured

in the absence of NS-398 ($P < 0.05$ for each).

A sample of the cultured cells were examined by electron microscopy, as described in Chapter 2.6. The cells cultured without NS-398 showed augmented nucleus and nucleolus, and altered ratio of nucleus to cytoplasm (Figure 5-8A). In contrast, the TE-13 cells cultured with NS-398 showed nuclear chromatin margination, nucleus pyknosis and cytoplasm condensation (Figure 5-8B).

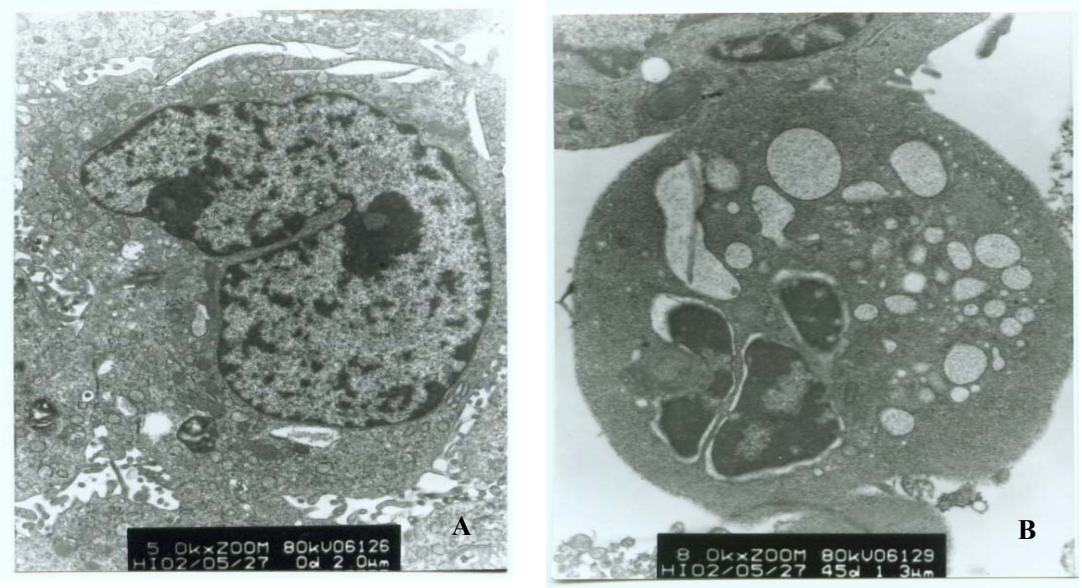

Figure 5-8. Representative electron micrographs of cultured TE-13 cells. (A) A TE-13 cell grown in the absence of NS-398 showed augmented nucleus and nucleolus and altered ratio of abnormal nucleus to cytoplasm (original magnification 6,000×) (B) A TE-13 cell grown in the presence of NS-398. Nuclear pyknosis approaching karyorrhexis is noted (original magnification 5,000×).

5.2.2.2. Effect of NS-398 on $PGF_{1\alpha}$ production

The concentration of $PGF_{1\alpha}$ in the supernatant of TE-13 cells incubated for 48 hours in the absence of NS-398 was 40.1 ± 5.7 pg/ml. In cells incubated for the same period with NS-398 added to the medium at a concentration of 0.001, 0.01, 1 or 100 μmol/L, $PGF_{1\alpha}$ production was significantly reduced to 27.4 ± 4.3 pg/ml, 24.9 ± 1.6 pg/ml, 24.7 ± 6.9 pg/ml and 24.1 ± 3.6 pg/ml respectively ($P < 0.05$ for each).

5.2.2.3. Effect of NS-398 on COX-2 mRNA expression

The level of COX-2 mRNA in the cells was measured semiquantitatively by end-point

PCR. The results in Figure 5-9 show that cells cultured with 0.001, 0.01, 1 or 100 μmol/L of NS-398 for 24 hours had 30.7%，31.8%，46.4% and 82.3% less transcript compared to cells without the drug ($P < 0.05$).

5.2.2.4. Effect of NS-398 on COX-2 protein expression

The results in Figure 5-10 show the effect of NS-398 on the intracellular concentration of COX-2 as measured by Western blotting. Incubation of the cells for 24 hours in medium to which either 0.001, 0.01, 1 or 100 μmol/L of NS-398 had been added each resulted in a significant decrease of 21.9%，24.0%，45.4% and 47.0% respectively in COX-2 protein expression. The results in Figure 5-11 show that there was a significant reduction over time in the COX-2 protein concentration in cells harvested between 60 minutes to 48 hours following the addition of 1 μmol/L NS-398 to the culture medium.

5.2.2.5. Effect of NS-398 on NF-KappaB binding activity

The presence of NF-KappaB in the nuclear extract was determined by its binding to its consensus sequence in an EMSA. Binding specificity in the reaction was confirmed with the use of homologous (NF-KappaB) and nonhomologous (AP-2) oligonucleotides as competitors (Figure 5-12C). The results in Figure 5-12A show that NF-KappaB was present in the control cells. Cells incubated for 24 hours with either 0.01, 1 or 100 μmol/L of NS-398 each showed a reduction in the amount of NF-KappaB. To determine the time course of this reduction, cells were incubated with 1 μmol/L of NS-398 and harvested at various time points up to 12 hours thereafter. The results in Figure 5-12B show that the reduction was induced within 30 minutes of the addition of the drug.

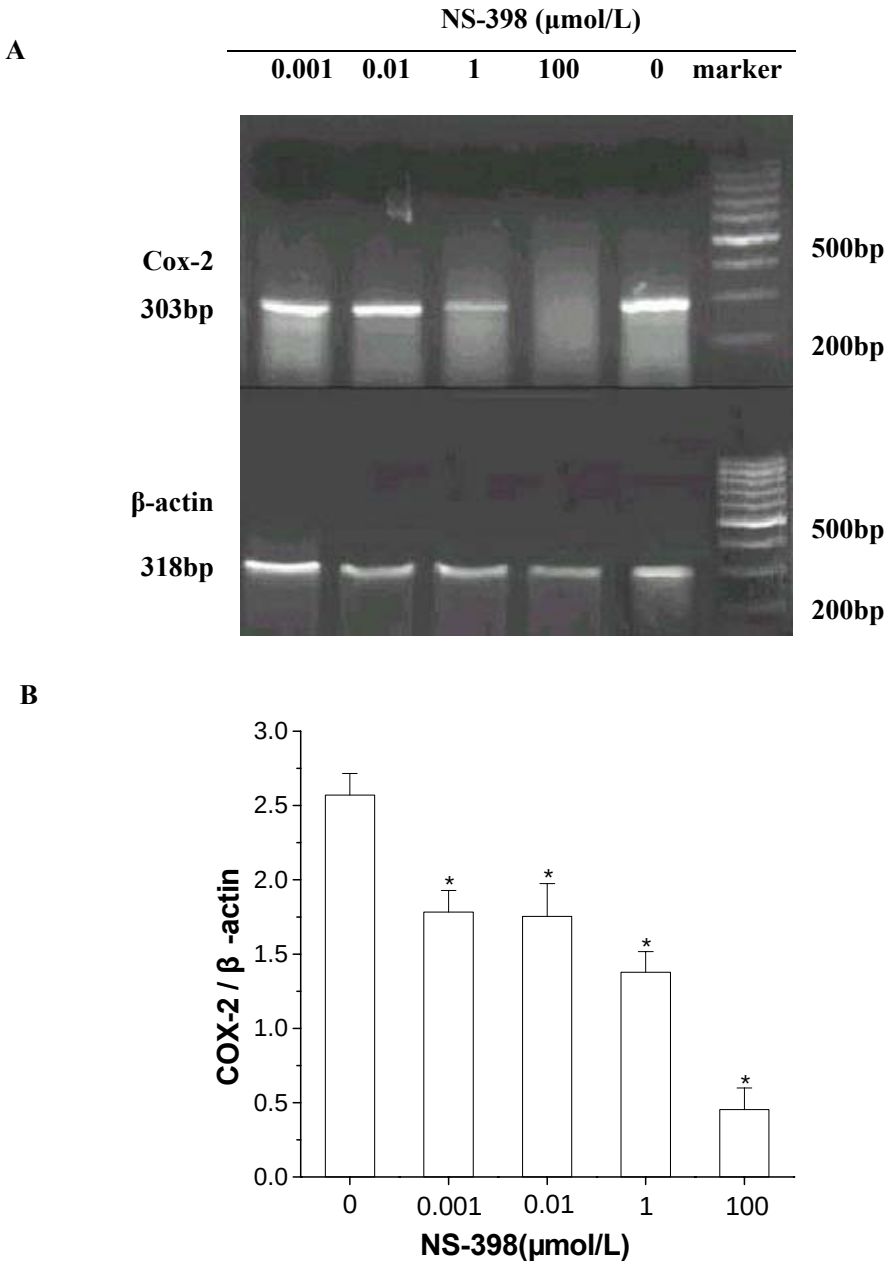

*Figure 5-9. The effect of the concentration of NS-398 on the inhibition of COX-2 mRNA expression measured by RT-PCR in TE-13 cells. (A) A representative gel of COX-2 and beta-actin mRNA when NS-398 was added at different concentrations. (B) COX-2 expression relative to expression of beta-actin, quantitated by Gel-Pro analyzer software (mean ± SEM of four independent experiments; * $P < 0.05$ compared to 0 mmol/L).*

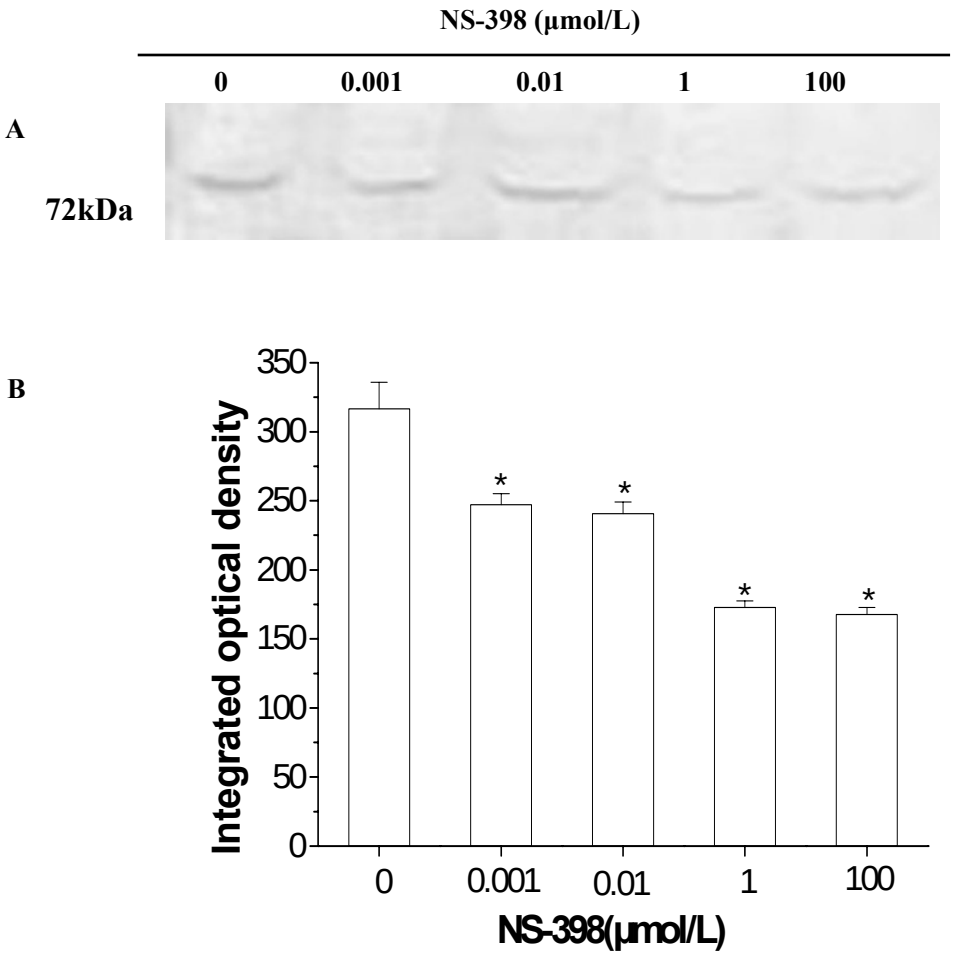

*Figure 5-10. The effect of the concentration of NS-398 on the inhibition of COX-2 protein expression measured by Western blotting in TE-13 cells. (A) A representative gel of COX-2 protein concentration when aspirin was added to the cultures at the concentrations indicated. (B) COX-2 expression quantitated by Gel-Pro analyzer software (mean ± SEM of four independent experiments; * $P < 0.05$ compared to 0 mmol/L).*

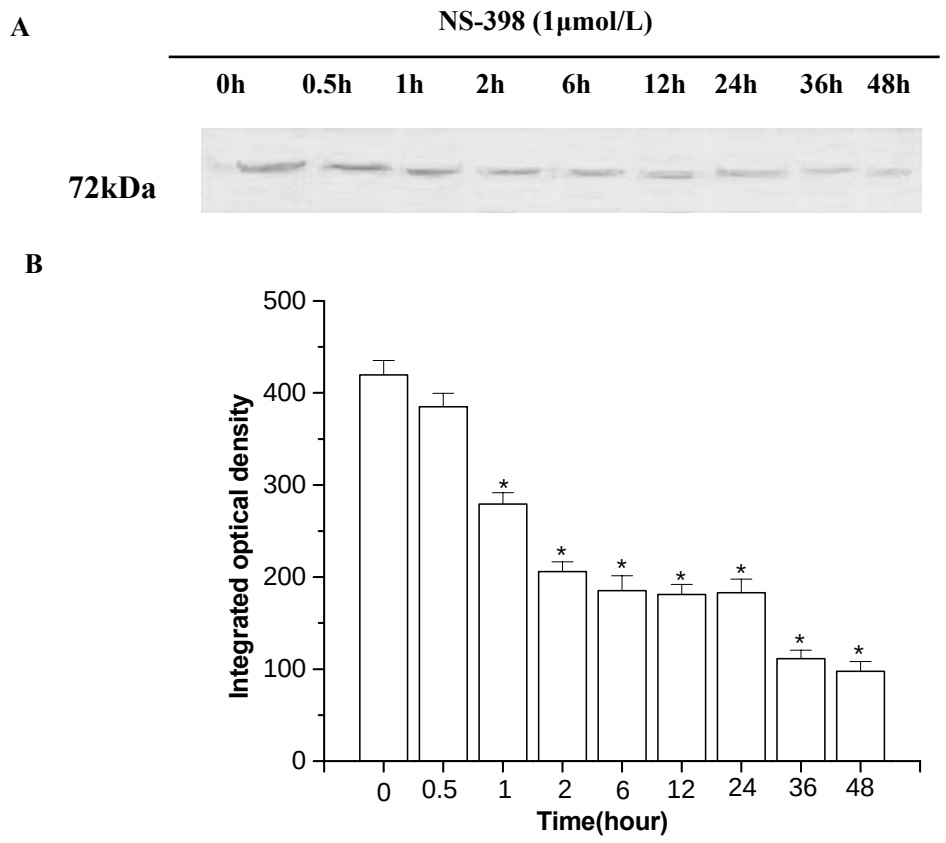

*Figure 5-11. The effect of time on the inhibition by NS-398 of COX-2 protein expression measured by Western blotting in TE-13 cells. (A) A representative gel of COX-2 protein concentration when aspirin was added to the cultures for the times indicated. (B) COX-2 expression quantitated by Gel-Pro analyzer software (mean ± SEM of four independent experiments; * $P < 0.05$ compared to 0 hours).*

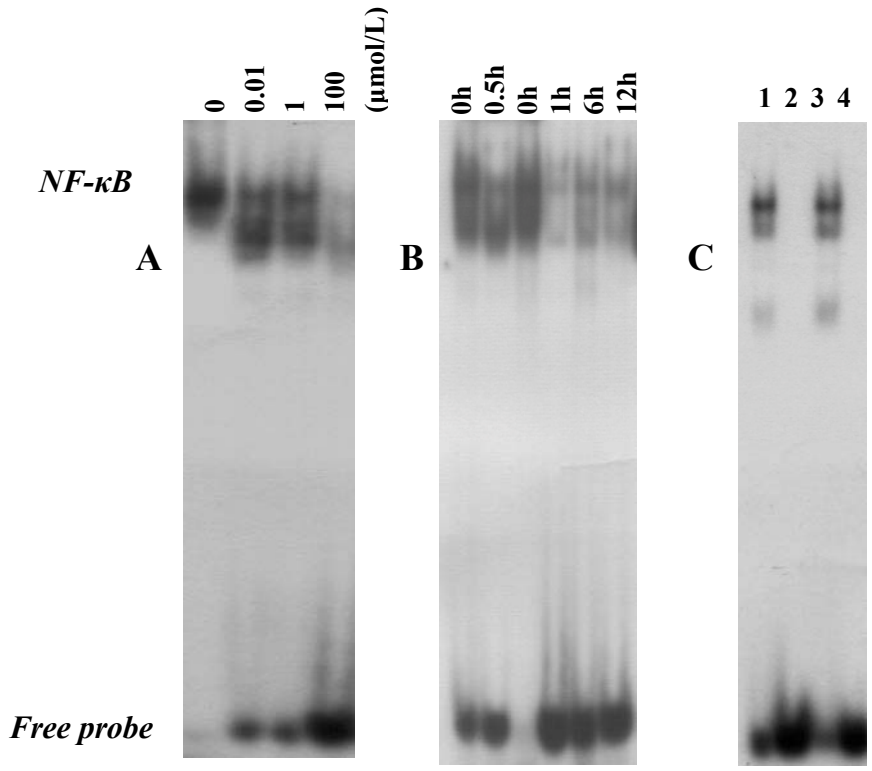

Figure 5-12. The activity of NF-κB measured with EMSA in TE13 cells treated with NS-398. (A) NF-κB binding activity in TE13 cells treated with NS-398 at different concentrations for 24 hours. (B) NF-κB binding activity in TE13 cells treated with NS-398 at 1μmol/L for different time. (C) The binding specificity was confirmed by using homologous (NF-κB) and nonhomologous (AP-2) oligonucleotide as competitors. lane 1, positive control; lane 2, negative control; lane 3, 32P-labeled Oligo plus unlabeled AP-2 Oligo (noncompetitor); lane 4, 32P-labeled NF-κB Oligo plus unlabeled NF-κB Oligo (competitor).

5.2.2.6. Effect of NS-398 on I-KappaB protein level

The results in Figure 5 show the effect of NS-398 on the intracellular concentration of IKappaB as measured by Western blotting. Incubation of the cells for 24 hours in medium to which either 0.001, 0.01, 1 or 100 μmol/L of NS-398 had been added each resulted in a significant increase of IKappaB protein level (Figure 5-11). The results in Figure 5-13 show that there was a significant increase in the IKappaB protein concentration at all time points between 30 minutes and 48 hours following the addition of 1 μmol/L of NS-398 to the culture medium.

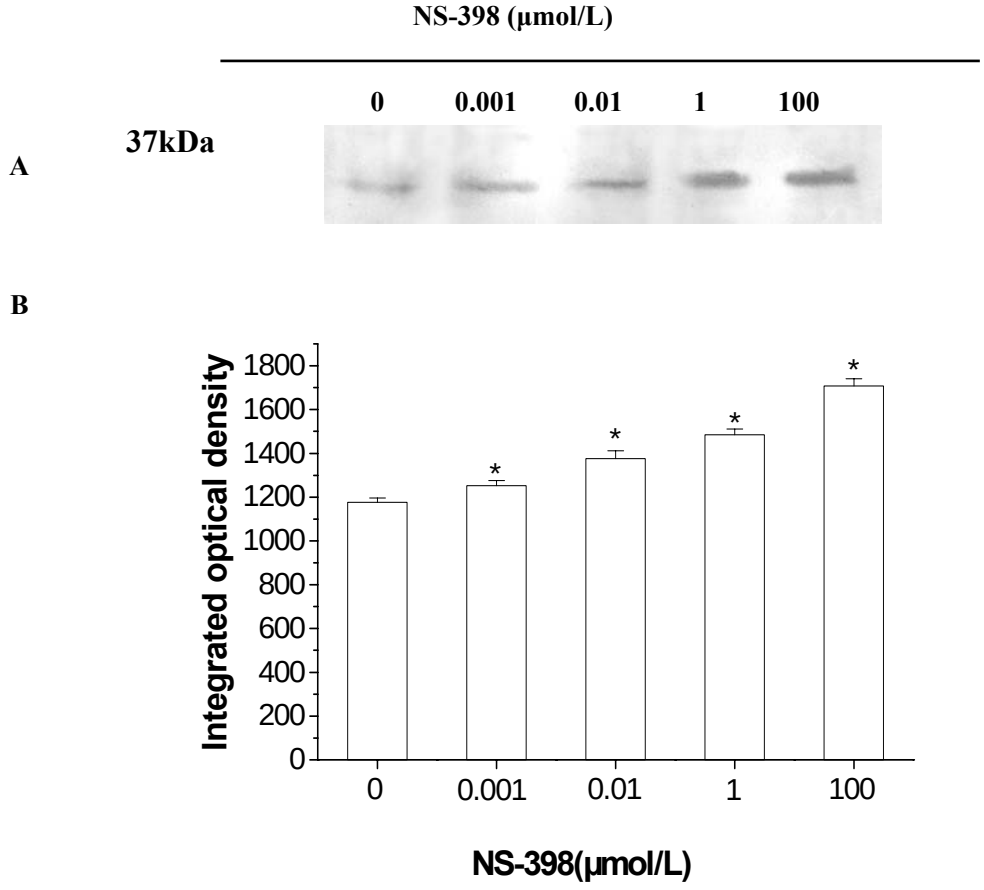

*Figure 5-13. The effect of the concentration of NS-398 on the IKappaB protein level in the cytoplasm of TE-13 cells measured by Western blotting. (A) A representative gel of IKappaB protein concentration when NS-398 was added to the cultures at the concentrations indicated. (B) IKappaB expression quantitated by Gel-Pro analyzer software (mean ± SEM of four independent experiments; * $P < 0.05$ compared to 0 µmol/L).*

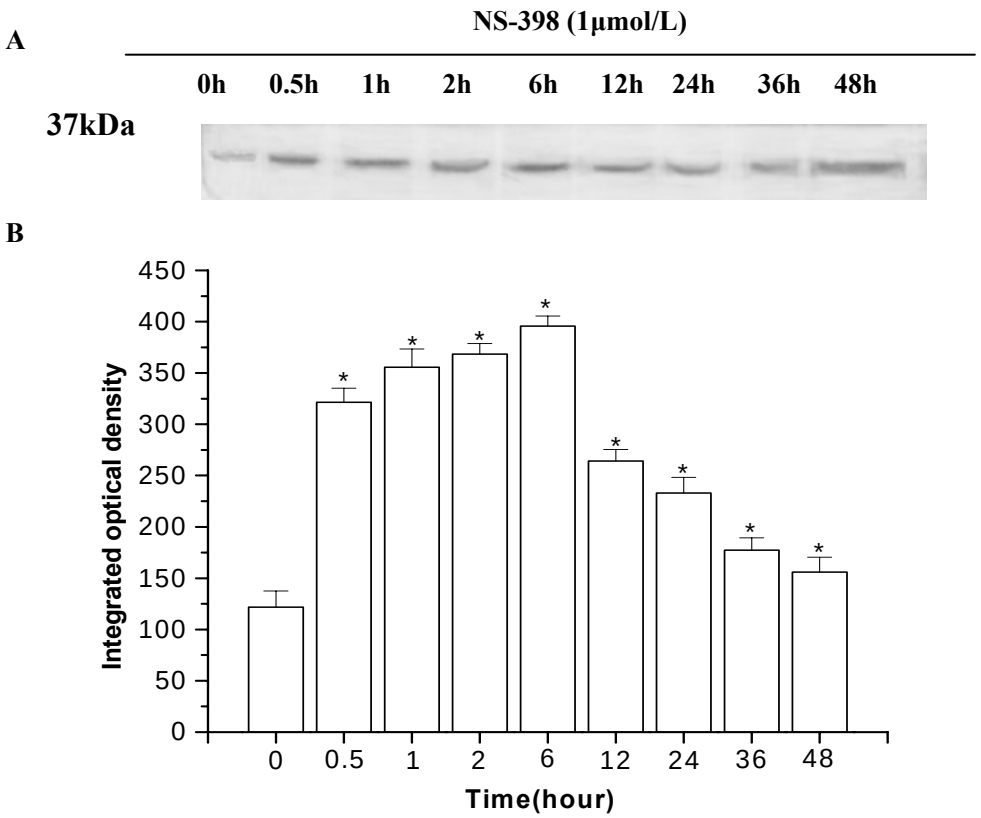

*Figure 5-14. The effect of time on the inhibition by NS-398 of IKappaB protein level in the cytoplasm of TE-13 cells measured by Western blotting. (A) A representative gel of IKappaB protein concentration when aspirin was added to the cultures for the times indicated. (B) IKappaB expression quantitated by Gel-Pro analyzer software (mean ± SEM of four independent experiments; * $P < 0.05$ compared to 0 hours).*

5.3 Conclusions

The results reported in this Chapter show that aspirin and NS-398 inhibited the proliferation of, and induced apoptosis in, the cultured SCC cell line TE-13. These changes correlated with a reduction in COX-2 mRNA and protein expression, prostaglandin synthesis, and an inhibition of NF-kappaB nuclear translocation, and an increase in cytoplasmic IKappaB. These results support the further study of the cyclooxygenase pathway in order to investigate the potential and mechanism of action of aspirin and similar drugs in cancer prevention and therapy.

Chapter 6. The effects of a selective COX-2 inhibitor, meloxicam on oesophageal squamous cell cancer tissue *in vivo*

6.1. Introduction

The results in the earlier chapters showed that both aspirin and NS-398 reduced the proliferation of oesophageal cancer cells, and induced apoptosis, in a dose dependent manner *in vitro*. These effects correlated with a reduction in COX-2 expression and prostaglandin synthesis, an increase in IKappaB and a reduction of NF-KappaB and COX-2 mRNA. The purpose of this study was to determine if similar changes occurred *in vivo* in the tumours of patients with SCC of the oesophagus who were given a selective COX-2 inhibitor, meloxicam.

6.2. Patients

Fifty three patients who had an oesophagectomy in the Department of Thoracic Surgery at the Fourth Hospital of Hebei Medical University for SCC were allocated randomly to either a Treatment group (n = 25) or Control group (n = 28). The patient information is given in Table 6-1. There were no significant differences in sex, age, stage of disease, or tumour differentiation between the groups. No patients received chemotherapy, radiotherapy or any other cancer related therapies before surgery. Patients in the Treatment group were given 7.5 mg per day of the selective COX-2 inhibitor meloxicam, marketed as Mobic (Boehringer Ingelheim), starting between 10 to 14 days before surgery, depending on the time interval between their admission and their operation. They stopped the drug the day before operation. No side effects which could be attributed to the drug were noted in the Treatment group. Patients in the Control group did not take any type of NSAID during this time interval between admission and operation. Samples of the tumour were taken from the resection specimens of each of the patients at oesophagectomy, snap frozen in liquid nitrogen, and then stored at -80^{o}C until analysis. Five ml of venous blood was collected into the anticoagulant indomethacin-sodium EDTA (the standard

anticoagulant used in the hospital). The blood was immediately centrifuged at 3,500 rpm for 15 min at 4°C, and the plasma collected and stored at -20°C.

The presentstudy was approved by the Ethics Committee, the Fourth Hospital, Hebei Medical University, China, and informed consent was obtaind from all patients.

Table 6- 1. Clinicopathologic characteristics of the patients in the study of the effect of meloxicam, a selective COX-2 inhibitor, on tumour cells in vivo.

	Treatment	Control
N	25	28
Sex m/f	19/6	21/7
Age (mean)	58.8	60.1
Age (range)	38-71	35-75
TNM stage		
IIb	20	22
III	5	6

6.3. Results

6.3.1. The effects of meloxicam on the expression of COX-2 protein

The amount of COX-2 protein in the oesophageal tissue was measured by Western blotting (Chapter 2.9). Weak expression of COX-2 was seen in 16 tumours and no expression in 9 tumours from patients in the Treatment group. In a representative gel shown in Figure 6-1 there is weak expression (lanes 1, 5 and 6) or no expression (lane 2) in tissue from 4 patients in the Treatment group (T), and strong expression in tissue from 2 patients (lanes 3 and 4) in the Control group (C). The amount of COX-2 in each subject, measured as Integrated Optical Density Units (IOD; as described in Chapter 2.9), is shown in Figure 6-2. The amount of COX-2 in the Treatment group (median 7.6) was significantly less than that in the Control group (median 28.3) (Mann Whitney U test, $P < 0.0001$).

6.3.2. The effect of meloxicam on prostaglandin concentration in plasma and in oesophageal cancer tissue

To determine if the reduction in COX-2 expression resulted in a reduction in prostaglandin, the concentration of 6-keto-prostaglandin F1α (6-Keto-PGF1α) was

measured in the plasmas and the resected tumour tissues (Chapter 2.7). This prostaglandin was measured as a representative prostaglandin as it was the one for which the necessary reagents, in particular the specific antibody, were readily available.

The results in Figure 6-3 show that the concentration of 6-keto-PGF1α was significantly lower in the tumour taken from patients in the Treatment group (median 9 pg/mg) compared to that from the Control group (median 28 pg/mg) (Mann-Whitney U test, $P < 0.001$). This reduction in concentration in the Treatment group was due in the main to the inhibition of COX-2 in the tumour by the drug, as the concentration of 6-Keto-PGF1α did not differ between plasma samples taken after treatment compared to samples taken before treatment (median of each was 90 pg/ml).

6.3.3. The effects of meloxicam on the expression of COX-2 mRNA

To determine if the reduction in COX-2 protein expression was the result of a reduction in its gene expression, the expression of COX-2 mRNA was measured in the resected oesophageal tumour tissue by RT-PCR (Chapter 2.8). A gel of the amplified products from a representative RT-PCR is shown in Figure 6-4. The lower are the housekeeping beta-actin bands, used as a common reference for comparing the results from the different tissues, and the upper are the COX-2 bands. The left row is from a patient in the Control group, the next 4 rows to the right were from 4 patients in the Treatment group. There is a prominent COX-2 band for the tissues from the Control group, while tissues from the Treatment group show weak COX-2 bands.

The optical density of each of the bands was quantitated and the ratio of IOD of the COX-2 band to the IOD of the beta-actin band was calculated. The results are shown in Figure 6-5. The median ratio for the Treatment group (0.57) was significantly lower than the median for the Control group (0.86) (Mann Whitney U test, $P < 0.0001$), indicating that the COX-2 gene expression was less in the tumour of patients in the Treatment group than in the Control group.

6.3.4. The effects of meloxicam on activity of NF-KappaB in SCC of the

oesophagus

One possible reason for the observed reduction in COX-2 mRNA would be an alteration in the level of the NF-KappaB transcription factor, which regulates COX-2 expression in at least some cell lines. The level of NF-KappaB in the tumour tissue was measured by electrophoretic mobility shift assay (EMSA) as described in Chapter 2.10. A gel from a typical EMSA assay is shown in Figure 6-6. In this figure the activity of NF-KappaB in the lanes 2, 3, 4 and 6, each from a tissue from a Treatment group patient, is greater compared to tissue from a Control group patient (lanes 1, 5, 7 and 8). The density of the bands in the EMSA gels was quantitated as IOD units. These results are shown in Figure 6-7. The median IOD for the Treatment group (8) was significantly less than that for the Control group (39) (Mann Whitney U test $P < 0.0001$).

6.3.5. The effects of meloxicam on the expression of IKappaB-alpha protein

One possible reason for a reduction in the level of NF-KappaB in the Treatment group might be an increase in its inhibitor, IKappaB-alpha. Western blotting was used to detect IKappaB-alpha in the tumour tissues resected from patients in the Treatment and the Control groups (Chapter 2.9). A gel from a typical Western blot is shown in Figure 6-8. In this gel, in the Control group there was either a weak band (lanes 1 and 5) or no band (lanes 2 and 4), while in the Treatment group the bands were prominent (lanes 3, 6 and 7). The density of the bands in the gels was quantitated as IOD units. These results are shown in Figure 6-9. The median IOD for the Treatment group (153) was significantly greater than that for the Control group (75) (Mann Whitney U test $P < 0.0001$).

6.3.6. The effects of meloxicam on proliferation and apoptosis in squamous cell carcinoma of the oesophagus

These experiments were undertaken to determine the effect of meloxicam on the proliferation and apoptosis of tumour cells in SCC of the oesophagus. The level of proliferating cell nuclear antigen (PCNA), expressed as the fluorescence index (FI) of cells stained with fluorescein conjugated antibody, was used as a measure of cell division (Chapter 2.2). Apoptosis was calculated as an apoptotic rate (AR) from

flow cytometry on cells stained with propidium iodide (PI) (Chapter 2.5). The results in Table 6-2 show that there was no difference in the percentage of PCNA positive cells between the tumours from patients who were on meloxicam and those who were not.

Table 6-2. The FI value of PCNA and apoptotic rate in SCC of the oesophagus by FCM

	FI of PCNA	AR (%)
Treatment (N = 15)	1.23±0.1	6.2±2.5
Control (N = 20)	1.32±0.1	1.2±1.1
P	0.16	0.0005

The histogram for a typical analysis of PI stained cells is shown in Figure 6-10, with the peaks labelled. The major peak is due to the cells in G0/G1, the peaks to the right are cells later in the division cycle, and the peak to the left of the G0/G1 peak is due to the cells undergoing apoptosis. The apoptotic rate (AR) is the ratio of the number of events in the apoptotic peak to the total number of events expressed as a percentage. Typical histograms of cell preparations from tumours from a patient in each of the Treatment and the Control group are shown in Figure 6-11.

The results in Table 6-2 show that there were significantly more apoptotic cells in the tumours of patients who were using meloxicam (mean 6.2%) compared to those who were not using meloxicam (mean 1.2%) (P = 0.0005).

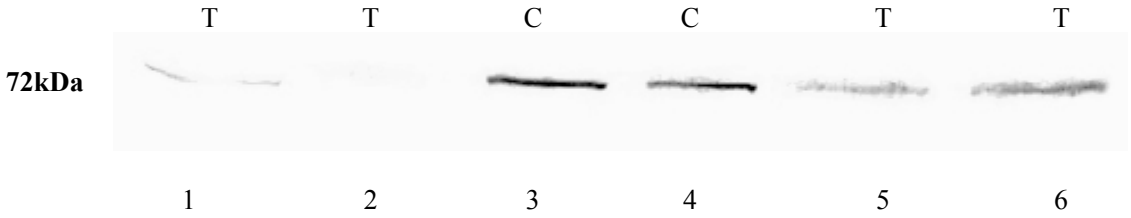

Figure 6-1. Western blot for COX-2 expression in SCC of the oesophagus. T = Treatment group (tumour tissue from patients who took meloxicam before surgery); C = Control group (tumour tissue from patients who took no NSAIDs before surgery).

.

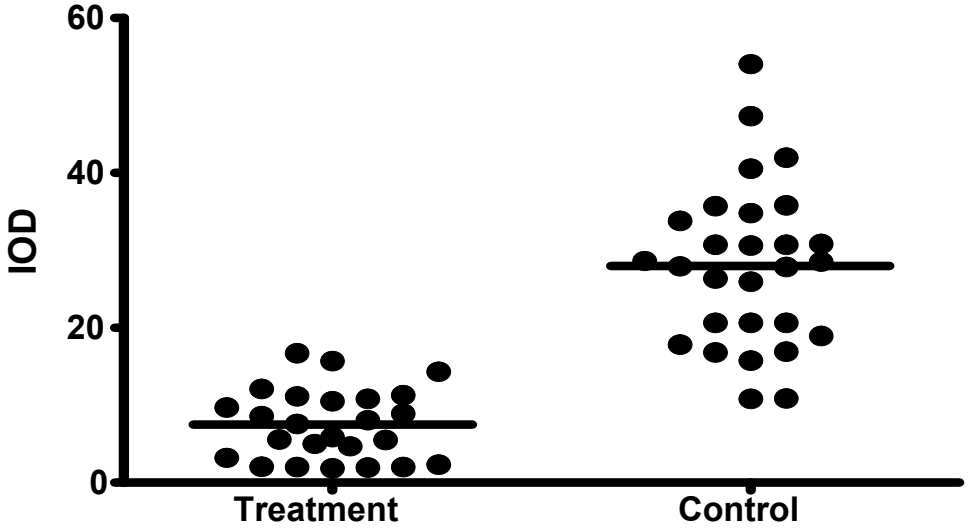

Figure 6-2. The amount of COX-2 in resected tumour tissue in each subject from the Treatment and the Control groups, as measured by Western blotting, expressed as integrated optical density units (IOD). Each point represents a different individual. The horizontal bars represent the medians of the groups ($P < 0.01$).

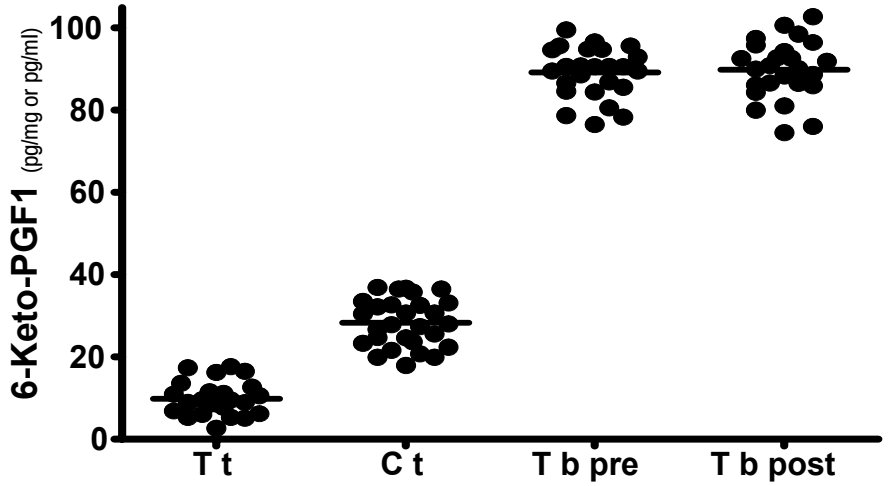

Figure 6-3. The concentration of 6-Keto-PGF1α in resected tumour issue from the Treatment group (Tt), or the control group (Ct) ($P < 0.01$), or the concentration in the plasma before (Tbpre) and after taking meloxicam (Tbpost) in the Treatment group ($P > 0.05$). Each point represents a different individual. The horizontal bars represent the medians of the groups.

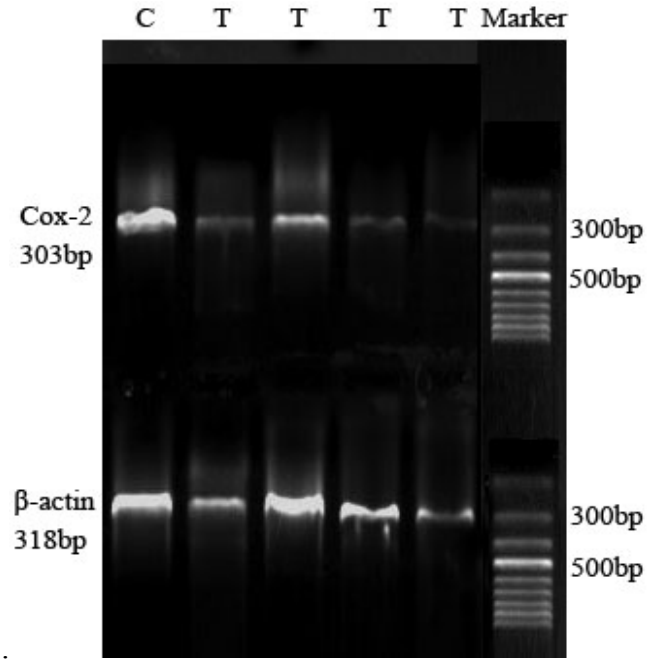

Figure 6-4. A representative gel from an RT-PCR assay for COX-2 mRNA in tissue from SCC of the oesophagus. The semi-quantitative analysis for COX-2 mRNA was performed by multiplex RT-PCR technique, using beta-actin as the reference standard. T = Treatment group (cancers from patients who used meloxicam before surgery); C = Control group (cancers from non-users of meloxicam).

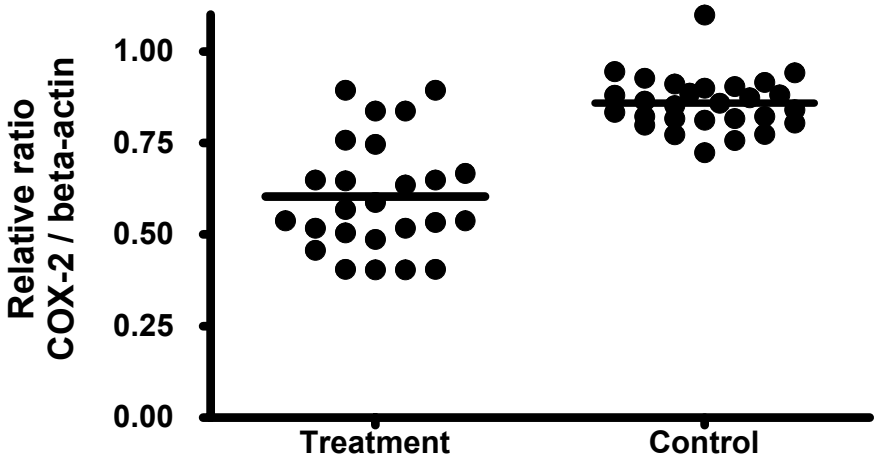

Figure 6-5. The relative expression of COX-2 mRNA determined by RT-PCR in tissue from SCC of the oesophagus. The relative expression is calculated as the ratio of the IOD of the COX-2 band to the IOD of the housekeeping gene beta-actin band. Each point represents a different individual. The horizontal bars represent the medians of the groups ($P<0.0001$).

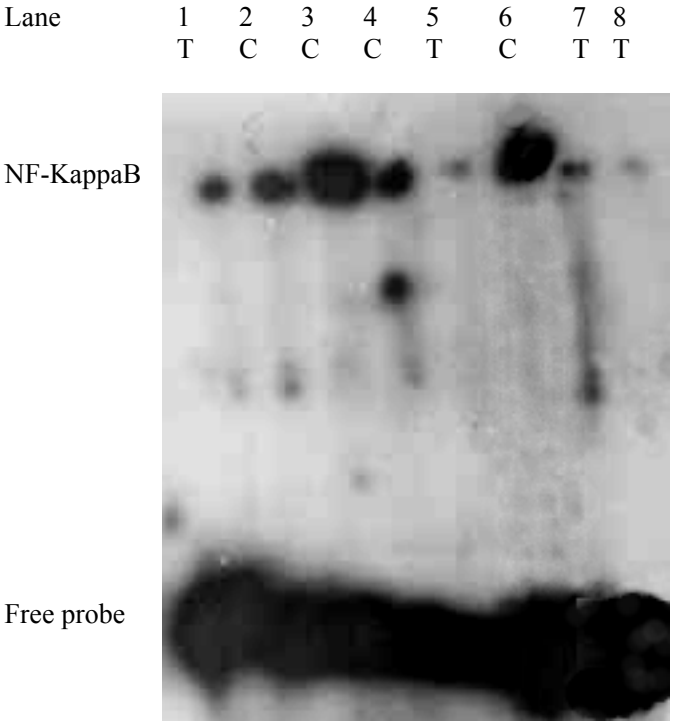

Figure 6-6. A gel from a representative EMSA assay for the determination of the activity of NF-KappaB in tumour tissues resected from patients with SCC of the oesophagus. Tissue from a patient in the Treatment group is denoted by T, from a patient in the Control group by C.

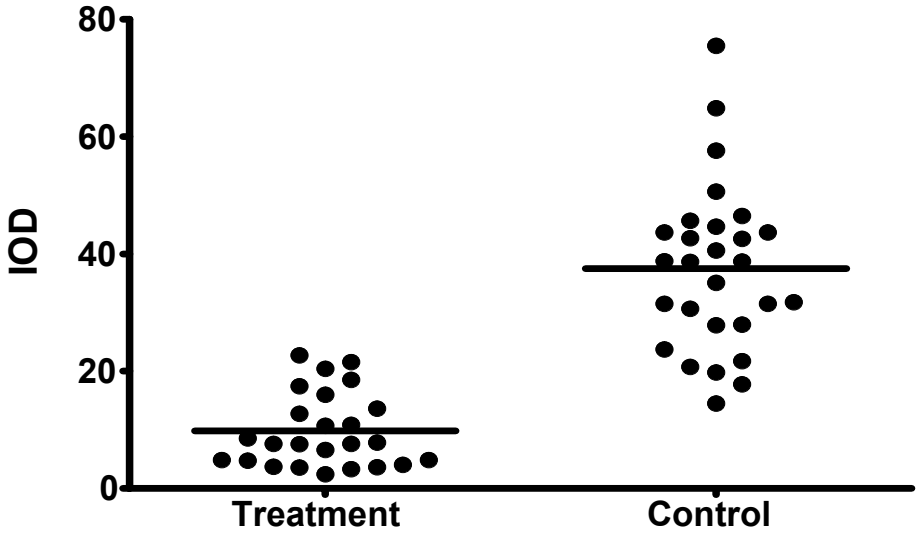

Figure 6-7. The IOD values for NF-KappaB as measured by EMSA in tissue from SCC of the oesophagus from patients in the Treatment and the Control groups. Each point represents a different individual. The horizontal bars represent the medians of the groups (P<0.0001).

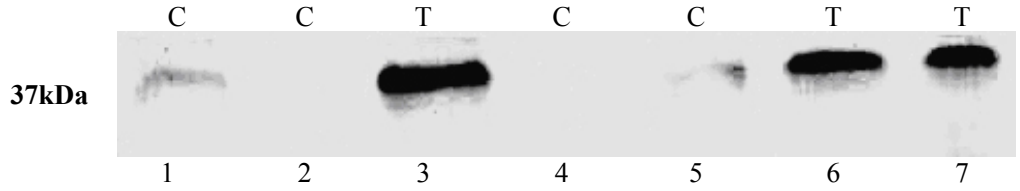

Figure 6-8. A representative Western blot for IKappaB-α protein levels in resection tissue from SCC of the oesophagus. T = Treatment group (tissues from patients who used meloxicam before surgery); C = Control group (tissues from patients who did not use meloxicam before surgery).

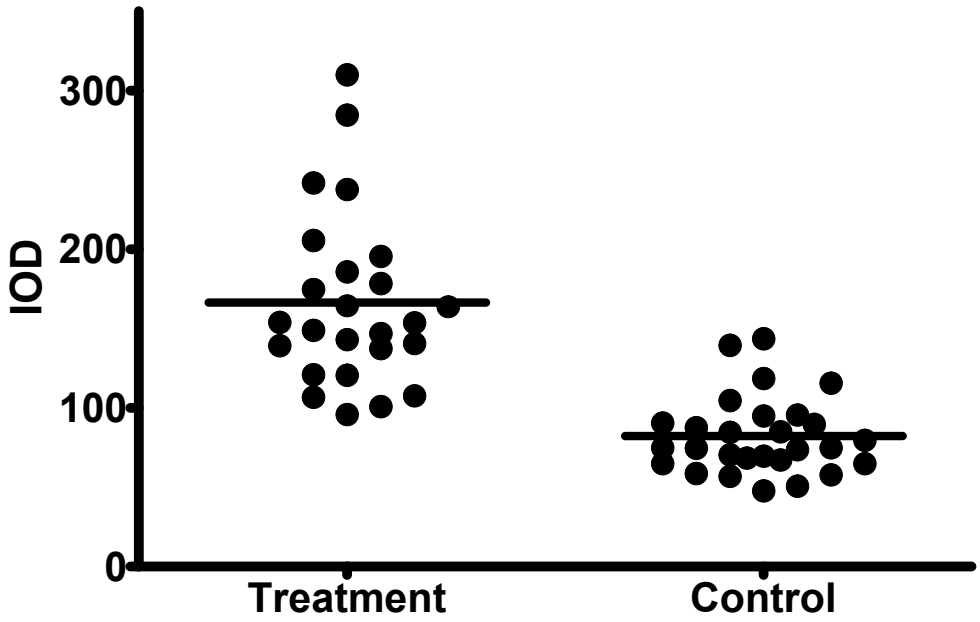

Figure 6-9. The IOD values for IKappaB-alpha as measured by Western blotting in tissue from SCC of the oesophagus from patients in the Treatment and the Control groups. Each point represents a different individual. The horizontal bars represent the medians of the groups (P<0.0001).

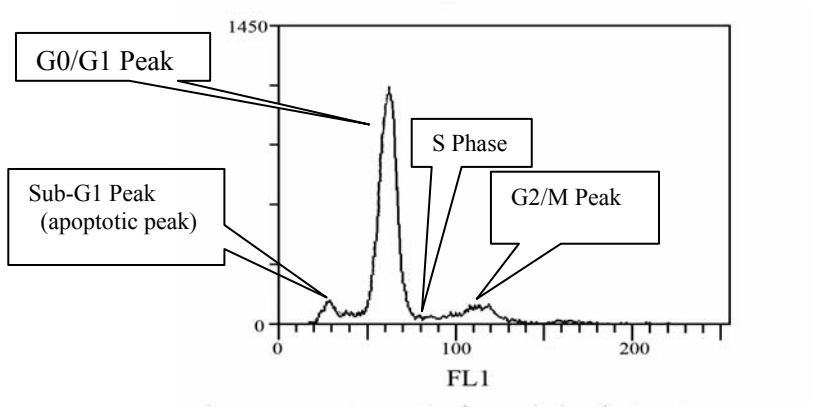

Figure 6-10. A histogram from a typical FACS analysis of PI-stained cells, with the peaks described.

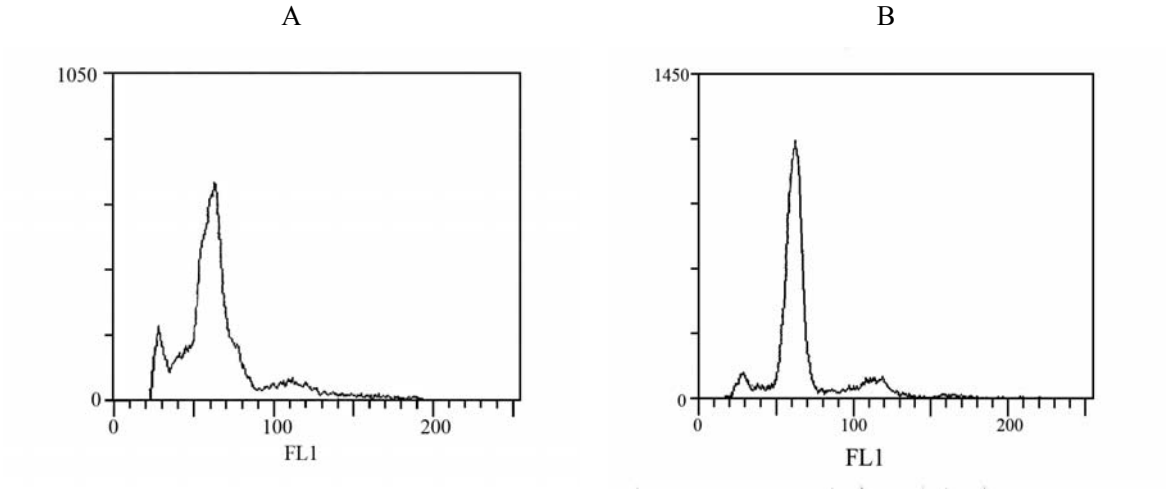

Figure 6-11. Representative FACS histograms of cells prepared from oesophageal SCC tumour tissue of a patient in the Treatment (A) and the Control (B) groups. The peaks are as described in Figure 6-10. The apoptotic rate in (A) is 10.6%, in (B) 5.5%.

6.4. Conclusions

This study demonstrates that in patients with oesophageal SCC given a selective COX-2 inhibitor, meloxicam, for between 10 to 14 days prior to operation, there was a reduction in the expression of COX-2 protein in the resected oesophageal SCC tissue. This was mirrored by a decrease in the concentration in the tumour tissue of the prostaglandin for which assay reagents were available, 6-keto-prostaglandin F1α. There was a reduction in COX-2 gene expression in the tumour tissue from the

treatment group, which paralleled a reduction in NF-KappaB activity and increase in the inhibitor IKappaB. In tumour from the Treatment group there was a reduction in PCNA levels, reflecting a reduction in the rate of cell division, and an increase in the number of cells undergoing apoptosis. Thus similar changes to those which COX-2 inhibition induced in tumour cells *in vitro* were seen in tumour tissue *in vivo* in patients given the selective COX-2 inhibitor meloxicam.

Chapter 7. The post-operative survival of patients given aspirin after resection for cancer of the oesophagus or gastric cardia

7.1. Introduction

Squamous cell carcinoma (SCC) is the most common type of oesophageal cancer in China (Li and Yao, 1997), while adenocarcinoma is the predominant type of cancer of the gastric cardia. The treatment of the 2 type of cancers is quite similar. Surgical resection provides excellent palliation, however the 5-year survival rate is less than 30% for SCC and less than 20% for adenocarcinoma. Adjuvant and neo-adjuvant therapies may result in an improvement in local control, but do not increase long-term survival. Thus the early diagnosis and effective treatment of these carcinomas is critical. While surgery continues as the mainstay of treatment, post-operative therapy may affect the rate or time of disease recurrence. Results presented in earlier chapters give hope that aspirin or NSAIDs might slow or prevent the growth of residual tumour following surgery. The aim of this study was to determine if aspirin provided any benefit to patients following oesophagectomy for cancer in terms of reduction in recurrence rate or increase in survival.

7.2. Study details

From May 2000 to December 2002, 1,716 patients underwent oesophagectomy for cancer in the Department of Thoracic Surgery, Fourth Hospital, Hebei Medical University. Standard adjuvant therapies as used in the institution were given to the patients: 5-fluorouracil (5-FU) for stage IIa or later stage adenocarcinoma of the gastric cardia, radiotherapy and cisplatin for stage IIa or later stage SCC of the oesophagus, or radiotherapy and VP-16 for undifferentiated carcinoma.

The patients were randomly allocated to one of three groups: group A (492 patients, given a daily dose of aspirin), group B (695 patients, given a placebo tablet daily) or group C (529 patients, given no tablets). Groups A and B started their tablets 14

days following the operation, and to be included in the study had to have taken them for at least 12 months following surgery. The daily dose of aspirin was 50 mg, reduced to 25 mg in patients with any gastric discomfort. Patients who underwent thoracotomy but the tumour was not resected due to its invasion into adjacent organs, and those who had a history of allergy to aspirin, were excluded from the study. Of the 1716 patients, follow-up was complete for 1676. Subsequent analysis was based on these patients only. The demographic and clinico-pathological characteristics of the three treatment groups are shown in Table 7-1. There were no significant differences between the groups with respect to the percentage of oesophageal or cardiac cancer, tumour location, disease stage, pathological type or the age or sex of the patients. The management of each patient remained with the responible surgeon. The design, management and analysis of the trial was the responsibility of the candidate.

7.3. Follow-up

All patients were monitored every 6 months until either the death of the patient or December 31, 2005. At each follow-up patients underwent a physical examination. Oesophagography and ultrasonography of the upper abdomen, gastroscopy, CT scan or isotopic skeletal scan were done if clinically indicated to detect any cancer recurrences or metastases. The incidence of cancer recurrences and metastases were recorded, and compared between the groups. Survival time was calculated from the day of surgery to death or until December 31, 2005. Survival analysis was carried out with a maximum follow-up of 67 months (mean follow-up, 40.8 months). The Kaplan-Meier method was used to calculate the survival curves, and statistical significance was assessed by the log-rank test. The survival analysis was corrected for cancer-related deaths only. Differences were considered to be statistically significant when the P value was equal to or less than 0.05.

Table 7-1 Demographic and clinico-pathological characteristics of the treatment and control groups.

Group	A	B	C
Treatment	Aspirin	Placebo	Nil
N	464	691	521
Sex m/f	353/111	507/184	380/141
Age (mean + SD)	58.2±8.3	58.5±8.2	58.3±8.2
TNM stage			
0	1	0	0
I	1	13	2
IIa	123	280	186
IIb	39	63	54
III	231	249	206
IVa	14	11	10
IVb	55	75	63
Site of tumour			
Upper third	25	41	32
Middle third	211	312	260
Lower third	54	60	44
Cardia	174	278	185
Histological type			
SCC	268	439	352
Adenocarcinoma	183	230	152
Squamo-adenocarcinoma	4	2	1
Undifferentiated	8	20	16
Carcino-sarcoma	1	0	0

7.4 Results

No side effect due to aspirin was noted in any patient in the study. This was anticipated because the dosage of aspirin lower than reported to cause side effects.

The survival curves for all patients who had a resection of the oesophagus for either cancer of the oesophagus or the gastric cardia are shown in Figure 7-1. The 5 year survival for Group A (aspirin) was 45.7%, for Group B (placebo) 37.8%, and Group C (no tablets) 37.4%. There was no significant difference in survival between any of the groups.

The effect of aspirin in patients with SCC of the oesophagus or adenocarcinoma of the gastric cardia was then analysed separately. The survival curves for patients operated on for SCC are shown in Figure 7-2. The 5 year survival for Group A (aspirin) was 46.2%, for Group B (placebo) 39.7% and for Group C (no tablets) 37.6%. There was no significant difference in survival between any of the groups.

The survival curves for patients operate on for adenocarcinoma of the gastric cardia are shown in Figure 7-3. The 5 year survival for Group A (aspirin) was 46%, for Group B (placebo) 33.4% and for Group C (no tablets) 38.8%. There was no significant difference in survival between any of the groups.

7.5. Conclusion

This study investigated the effect of a daily dose of aspirin *on* survival following oesophagectomy for cancer of the oesophagus or the cardia. There was a clear trend for longer survival in the group which took aspirin, although this did not reach statistical significance. The results from this trial support further investigation into the effect of other doses of aspirin, or of aspirin in combination with other therapies, or the value of NSAIDs, or of taking the drug for longer than the minimum of 12 months, as adjuvant therapy to improve survival in post-oeosophagectomy patients.

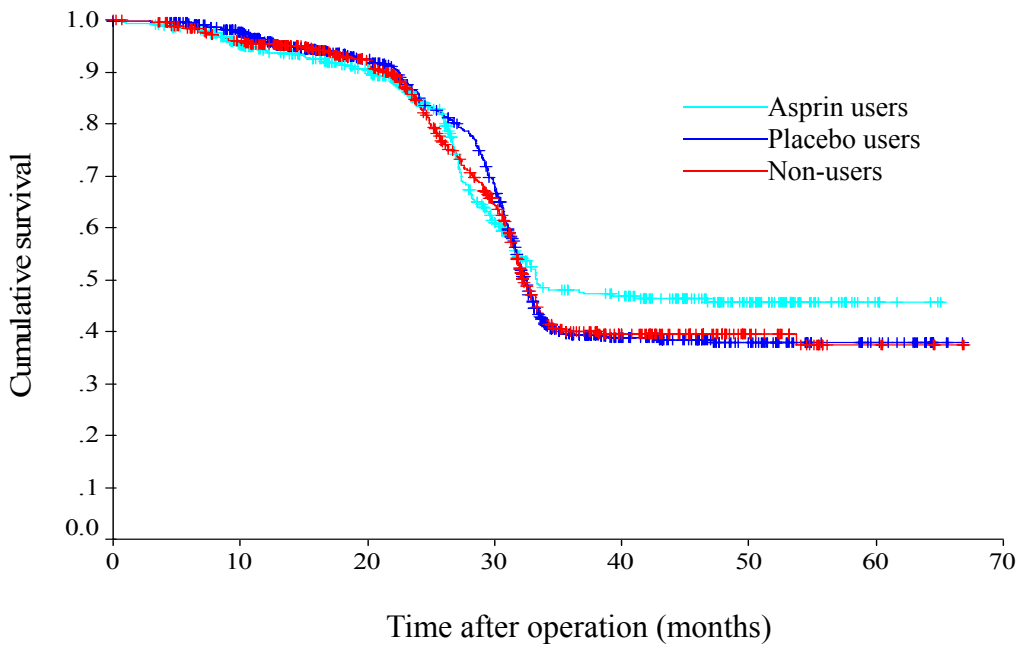

Figure 7-1. Kaplan-Meier survival curves for patients following resection of the oesophagus for SCC of the oesophagus or adenocarcinoma of the gastric cardia. The patients were randomly allocated to one of three groups: group A (464 patients, given a daily dose of 25-50 mg of aspirin), group B (691 patients, given a placebo tablet daily) or group C (521 patients, given no tablets). There was no significant difference in survival between the groups.

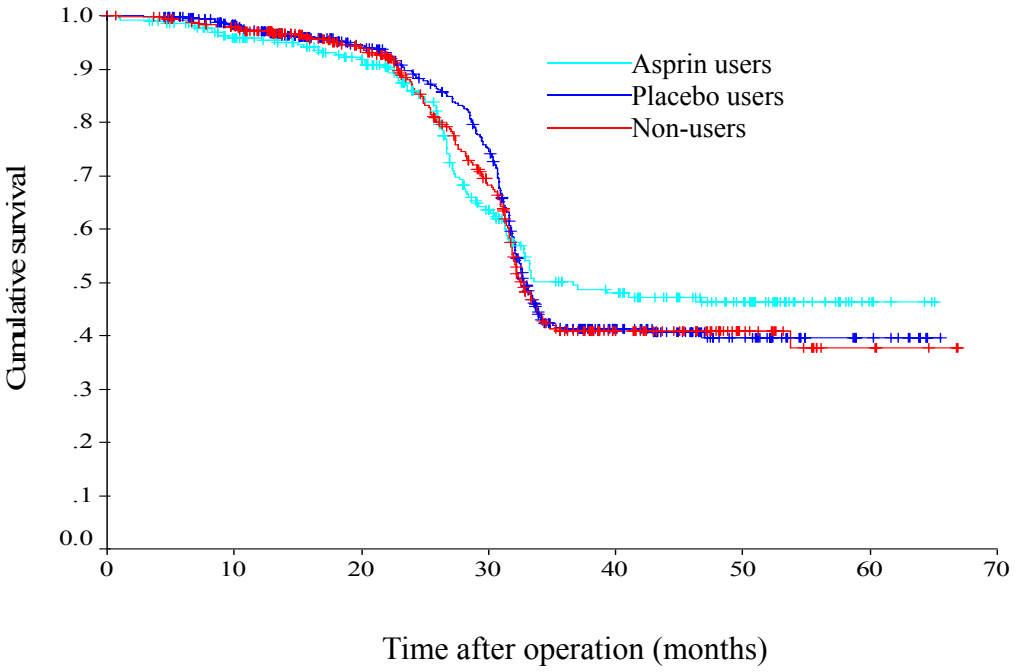

Figure 7-2. Kaplan-Meier survival curves for patients following resection of the oesophagus for SCC. The patients were randomly allocated to one of three groups: group A (264 patients, given a daily dose of 25-50 mg of aspirin), group B (439 patients, given a placebo tablet daily) or group C (352 patients, given no tablets). There was no significant difference in survival between the groups.

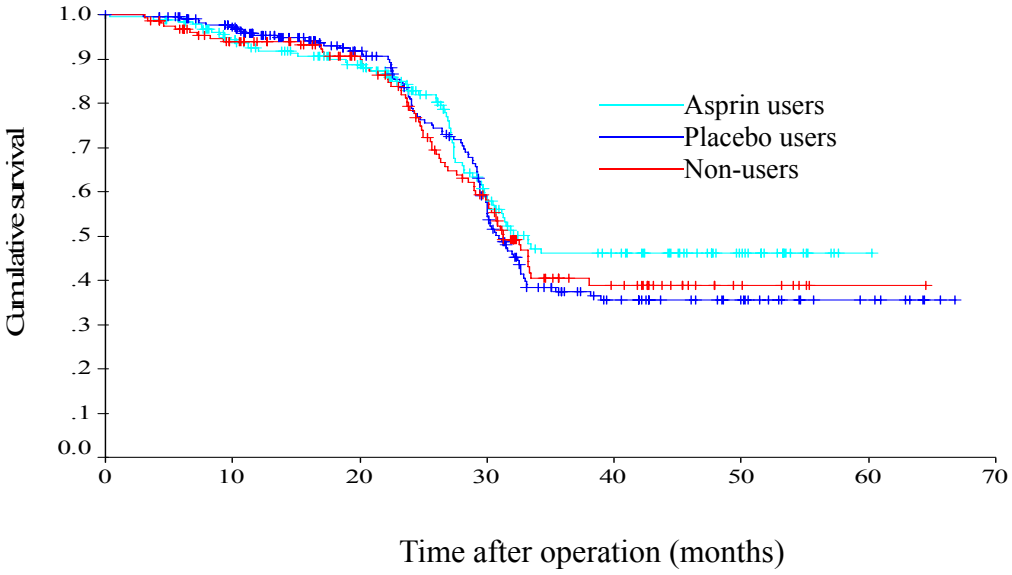

Figure 7-3. Kaplan-Meier survival curves for patients following resection of the oesophagus for adenocarcinoma of the gastric cardia. The patients were randomly allocated to one of three groups: group A (183 patients, given a daily dose of 25-50 mg of aspirin), group B (230 patients, given a placebo tablet daily) or group C (152 patients, given no tablets). There was no significant difference in survival between the groups.

Chapter 8. Discussion

8.1. Introduction

Oesophageal cancer is the seventh most frequent cancer worldwide (Pisani et al., 1993; Parkin et al., 1999) and the fourth most common malignancy in China (Li et al., 1996). It is an aggressive cancer, and although surgical treatment with adjuvant chemo-radiotherapy has been used in patients with this malignancy, the long-term survival is still disappointing. The 5-year survival rate has changed little over time, remaining at just over 20% (Li et al., 1996; Liu et al., 2004).

While the greatest improvement in long-term survival will come from strategies which target early diagnosis, improvement can also be expected from the development and appropriate application of other anti-cancer therapies. Epidemiological, experimental and clinical investigations indicate that aspirin and non-steroidal anti-inflammatory drugs (NSAIDs) have potential as chemopreventative agents in a variety of cancers, including oesophageal cancer. This thesis continues these investigations.

8.2. Squamous cell carcinoma of the oesophagus in Hebei Province, China

Most centres in the world manage only small numbers of patients with oesophageal cancer each year. Between September 1952, when the department was established, and December 2000, 15,653 patients underwent resection for cancer of the oesophagus and gastric cardia in the Department of Thoracic Surgery, Fourth Hospital of Hebei Medical University (Hebei Provincial Tumour Hospital), Shijiazhuang. Many of these patients have been reported on previously, but mainly in the Chinese literature without English translation. In Chapter 3 the surgical treatment and outcomes of the total cohort of patients over this period with carcinoma of the oesophagus and gastric cardia were reviewed. This is the largest single centre series of oesophagectomy ever reported on, and was made possible by the methodical recording and careful storing of the clinical notes by several generations of clinicians.

Over that time there was a gradual reduction in the postoperative morbidity and mortality rates, although the long-term survival of the patients remained disappointing. The long term survival rates were virtually identical to those reported in Western series. Surgical techniques changed little over the 50 year period, and improvements in surgical outcomes were mostly due to better post-operative care. Anastomotic leaks remain the major cause of operative morbidity and mortality. It was concluded that while improved results might be attained with more careful selection of patients for operation, and the development and rational use of other therapies, the greatest impact on long-term survival continues to be likely to stem from diagnosis of the disease at an earlier stage, together with improved adjuvant therapy.

8.3. COX-2 expression in SCC of the oesophagus

Increased expression of COX-2 protein, and its correlation with tumour stage and mortality has been reported in SCC of the oesophagus (Zimmermann et al., 1999; Nozoe et al., 2005), as well as many other cancers such as stomach (Zimmermann et al., 1999; Honjo et al., 2005), breast (Ristimaki et al., 2002), ovarian (Denkert et al., 2002), bladder (Shirahama et al., 2001) and lung (Mascaux et al., 2006). It has also been demonstrated that COX-2 is up-regulated in the progression from normal squamous epithelium through dysplasia to SCC of the oesophagus (Maaser et al., 2003). These findings, together with epidemiological evidence discussed in Chapter 1, suggest that COX-2 expression may be related to the development and progression of oesophageal cancer, and could be a useful indicator for prognosis in this disease, as well as a target for therapy.

In Chapter 4 the expression of COX-2 in SCC of the oesophagus was measured, and its significance as a prognostic indicator determined. The study group consisted of 90 men and 48 women who underwent oesophagectomy for squamous cell carcinoma of the oesophagus 15 years earlier. Expression was measured by immunohistochemistry (IHC) and by flow cytometry (FCM). The advantage of IHC is the ability to note the location within the cell, and distribution within the tissue, of the marker. The disadvantage is that the results at best are semi-quantitative, and based on the visualisation of a small number of cells. In contrast, FCM analyses a

large number of cells, and is quantitative, and it is more reliable to correlate the results with clinicopathological variables. However, no morphological information is available.

There was COX-2 expression detectable by IHC in tumour from 56% of the patients. In half of these cases with COX-2 detected by IHC the staining was patchily distributed through the tumour, in the other half the staining was evenly distributed throughout. Weak COX-2 staining was detected in 62% of normal oesophageal epithelia, with the COX-2 positive cells predominantly localized in the basal cell layer. There was COX-2 immunostaining in some muscle cells, mononuclear inflammatory cells, and vascular endothelial cells both in cancer infiltrated tissue and normal oesophageal tissue, as has been reported by others (Ratnasinghe et al., 1999; Zimmermann et al., 1999; Shamma et al., 2000). These findings reinforce reports of increased expression of COX-2 in oesophageal SCC measured by IHC (Zimmermann et al., 1999; Heeren et al., 2005), Western blotting (Zimmermann et al., 1999; Shamma et al., 2000; Zhi et al., 2003; Yu et al., 2004) and RT-PCR (Zhi et al., 2003; Yu et al., 2004). Recently polymorphisms in COX-2 have also been suggested to play a role in mediating susceptibility to cancer of the oesophagus and bladder (Kang et al., 2005; Zhang et al., 2005), indicating that both genotype as well as expression levels may play a role in the progression of certain tumours. Weak cytoplasmic staining of normal oesophageal squamous epithelium has been reported by others, but is not a universal finding (Zimmermann et al., 1999; Jiang et al., 2004). Differences in reported expression could be due in part to differences in sensitivity arising from variations in the antibodies used, in staining technique, or the grading of the staining between groups.

In contrast to the results using IHC, COX-2 expression was detectable in 95.7% of cancers by FCM, indicating that FCM was more sensitive than IHC. Well differentiated and moderately differentiated tumours had significantly stronger expression of COX-2 by FCM than poorly differentiated tumours, similar to a report by Ratnasinghe (Ratnasinghe et al., 1999). Hida (1998) reported markedly higher COX-2 expression in lymph node metastases of pulmonary adenocarcinoma compared to the primary tumour, but in this study there was no difference in expression between the primary tumour and the nodal metastases.

The relationship between COX-2 expression and a number of clinicopathological parameters was analysed. The grade of COX-2 expression as measured by IHC correlated positively with the finding of lymph node metastases. Thus lymph node metastases were more likely to be found in those patients whose primary tumour stained moderately or strongly for COX-2. Those patients with lower COX-2 expression survived significantly longer than those with higher expression, but that this survival could not be explained by the possibility that COX-2 staining was a surrogate marker for tumour stage. This is similar to oesophageal adenocarcinoma where France (2004) suggested that COX-2 expression might be a better prognostic indicator than traditional histopathological staging. The relationship between COX-2 expression and survival in oesophageal SCC remains unresolved. Two European studies, one with 81 patients, the other with 22, and a Taiwanese study with 96 patients, failed to find a relationship between COX-2 expression measured by IHC and outcome (Kuo et al., 2003; Heeren et al., 2005; Sivula et al., 2005). However a study of 76 Japanese patients reported that strong expression of COX-2 correlated with poor survival (Nozoe et al., 2005). This study, from a region of China with one of the highest incidences of SCC in the world, is the largest group of patients reported, and they were followed for longer than in any other reported study.

The expression of COX-2 is increased in oesophageal cancer and correlated with the finding of lymph node metastases. Importantly, higher levels of COX-2 expression were associated with shorter survival. The findings suggested that, at least for patients from this endemic region with a high expression of COX-2, therapeutic use of NSAIDs either before surgery, or after surgery to inhibit the development of metastatic disease, warranted investigation.

8.4. The effects of COX inhibitors on an SCC cell line

Aspirin and NSAIDs are inhibitors of COX enzymes. Their anti-cancer effect has been attributed to the inhibition of prostaglandin synthesis as a consequence of the inhibition of the COX-2 enzyme. Zimmermann (1999) showed that treatment with NS-398 (a COX-2 inhibitor) dose dependently suppressed PGE2 synthesis and cell proliferation, and also induced apoptosis in oesophageal cancer OSC-2 cell line,

which had constitutively high expression of COX-2. In contrast, NS-398 was without effect on the OSC-1 cell line, which is characterized by high levels of COX-1 but only weak expression of COX-2 (Zimmermann et al., 1999). Li *et al* (2001) reported that aspirin inhibited COX-2 activity (but not COX-2 protein level) and PGE2 synthesis in human oesophageal cancer cells, and growth inhibition of these cells by aspirin was dose- and time-dependent and associated with the induction of apoptosis. Several other studies, however, reported no correlation between COX-2 expression and apoptosis induced by NSAIDs, including NS-398 (Elder et al., 1997; Piazza et al., 1997; Zimmermann et al., 1999; Elder et al., 2000). Elder (2000) found no correlation between the sensitivity of a colon cancer cell line to NS-398 and COX-2 expression. Piazza (1997) reported that apoptosis induced by sulindac in cultured colon adenocarcinoma cells was independent of COX-2 inhibition, cell cycle arrest, and p53 induction. These studies suggest that NSAIDs can affect cells by mechanisms other than inhibition of the COX enzymes. The experiments in Chapters 5 and 6 were designed to explore further the effects of a non-selective COX inhibitor, aspirin, and a COX-2 specific inhibitor, NS-398, on tumour cells *in vivo* and *in vitro*.

The results in Chapter 5 show that the addition of either drug to cultures of the oesophageal SCC cell line TE-13 significantly inhibited cell proliferation and increased apoptosis. The COX-2 protein and mRNA expression were reduced by the treatment in a dose- and time-dependent manner. The concentration of PGF1α, a marker for prostaglandin synthesis, was reduced by the drugs. The findings of a dose-dependent inhibition of cell growth and induction of apoptosis in TE-13 cells are consistent with other reports. Li noted that aspirin induced apoptosis in both oesophageal squamous cell carcinoma and adenocarcinoma cell lines (Li et al., 2000), and the treatment of OSC-2 oesophageal cancer cell line with the selective COX-2 inhibitors, NS-398 and flosulide, suppressed proliferation and also induced apoptosis (Zimmermann et al., 1999). The dose range of aspirin used was 1 - 8 mmol/L, which corresponds to its pharmacological concentration *in vivo*. Together these results are consistent with the drugs inhibiting COX-2 induction and thereby the synthesis of prostaglandins, leading to growth inhibition and apoptosis.

Next one possible mechanism for the suppression of the transcriptional activation of

COX-2 was investigated. It has been reported that NF-KappaB, an inducible transcription factor, plays an important role in cell proliferation, transformation, and tumour development (Lim et al., 2001). Human and murine COX-2 gene promoters contain a regulatory DNA sequence to which NF-KappaB binds, and COX-2 expression is regulated by NF-KappaB in several cell lines (Siebenlist et al., 1994; Wulczyn et al., 1996). In cells NF-KappaB is normally located in the cytoplasm as a hetero- or hemo-dimer, and is non-covalently associated with cytoplasmic inhibitory proteins, including IKappaB (Lim et al., 2001). The IKappaBs can be phosphorylated by an activated IKappaB kinase (IKK) complex generated in response to a variety of stimuli, such as cytokines (TNF-alpha or IL-1) or by-products of bacterial or viral infections (endotoxin or double stranded DNA). Phosphorylation of IKappaBs results in their rapid ubiquitin-dependent degradation, freeing the NF-KappaB to translocate to the nucleus where it stimulates the expression of its target genes.

Pierce (1996) found that both aspirin and sodium salicylate inhibited NF-KappaB by preventing IKappaB phosphorylation and degradation, and Yin (1998) reported selective inhibition of the IKappaB kinase β (IKK-β) subunit and consequently NF-KappaB activity by aspirin at pharmacological concentration of 1 - 5 mmol/L. Lim (2001) reported that gastric cancer AGS cell line expressing COX-2 had constitutive NF-KappaB in the nucleus, and that inhibition of NF-KappaB by NS-398 suppressed cell proliferation although it did not induce apoptosis. The inhibition of NF-KappaB in gastric cancer cells and breast cancer cell lines has been demonstrated to result in the inhibition of proliferation and the induction of apoptosis (Sovak et al., 1997; Lim et al., 2001). But whether NSAIDs have similar effects on oesophageal cancer cells is unknown. The results in Chapter 5 show that aspirin and NS-398 each reduced the intracellular level of NF-KappaB as measured by EMSA, and also increased the level of IKappaB.

There was NF-KappaB expression in the nucleus of the oesophageal cancer cells, similar to what has been reported in gastric cancer (Lim et al., 2001), hepatic stellate cells (Gallois et al., 1998), and a colon cancer cell line (Kojima et al., 2000). Treatment of the TE-13 cells reduced nuclear NF-KappaB levels at the same concentrations as reduced COX-2 expression, suggesting that inhibition of

NF-KappaB translocation might be the upstream mechanism of the inhibitory effect of aspirin on COX-2 transcriptional activation. The reduced nuclear translocation of the NF-KappaB was consistent with the dose- and time-dependent increase in IKappaB protein level observed in the cytoplasm of the cells following treatment. The maximal effect on both NF-KappaB activity and IKappaB protein level occurred at 6 hours. These results suggest that the inhibition of NF-KappaB activity by aspirin is related to the increase in IKappaB protein level in the cytoplasm.

These experiments showed that the drugs inhibited the proliferation of, and induced apoptosis in, the cultured TE-13 SCC cell line. These changes correlated with a reduction in COX-2 mRNA and protein expression, prostaglandin synthesis, and an inhibition of NF-KappaB nuclear translocation and an increase in cytoplasmic IKappaB. This is believed to be the first report that aspirin and NS-398 inhibits human oesophageal cancer cells by inhibiting NF-KappaB activity, although it has been demonstrated in some studies on other cancer cells. These results support the further investigation of the potential of aspirin and similar drugs in cancer prevention and therapy.

8.5. The effects of a selective COX-2 inhibitor on SCC tissue *in vivo*

The results in Chapter 5 showed that drugs which inhibit COX activity also inhibited the proliferation of, and induced apoptosis in, the cultured SCC cell line TE-13. These changes correlated with a reduction in COX-2 mRNA and protein expression, prostaglandin synthesis, and an inhibition of NF-KappaB nuclear translocation and an increase in cytoplasmic IKappaB.

The experiments in Chapter 6 were designed to determine if the NSAID meloxicam (Mobic) had the same effect on oesophageal tumour cells *in vivo* as aspirin or NS-398 had on the oesophageal cell line TE-13 *in vitro*. Meloxicam, a selective COX-2 inhbitor structurally related to piroxicam, has a lower risk of adverse side effects than piroxicam, diclofenac, or naproxen. Importantly for this study, although it does inhibit thromboxane A, it is reported not to do so at levels that would interfere with platelet function.

The Treatment group of patients were given meloxicam for 10 to 14 days before surgery, the Control group took no NSAIDs for the same period of time. Samples of the tumour tissue and plasma were frozen immediately after resection and stored for subsequent analysis. There was a reduction in the expression of COX-2 protein in the resected oesophageal SCC tissue from the Treatment group. This was mirrored by a decrease in the concentration of prostaglandin in the tissue. The concentration of prostaglandin in the plasma did not differ between the two groups as would be anticipated. The plasma pool of prostaglandin reflects the production bodywide by the constitutive COX-1, which is not inhibited by meloxicam. There was reduction in the expression of COX-2 mRNA in the tumour tissue, which paralleled a reduction in NF-KappaB expression and an increase in IKappaB expression. There was also a reduction in PCNA levels in tumours from the Treatment group, consistent with an inhibition of cell proliferation caused by the meloxicam. In tissues from the Treatment group there were more apoptotic cells than in the Control tissues.

This is one of the few *in vivo* studies to examine mechanisms by which NSAIDs affect cancer tissues in general or oesophageal SCC tissue specifically. Yin (1998) demonstrated that aspirin and sodium salicylate specifically inhibited IKK-beta activity *in vitro* and *in vivo*, thereby preventing activation of NF-kappaB responsive genes. Sclabas *et al* (2005) reported that aspirin, given before or at the time of transplantation into mice of a human pancreatic carcinoma cell line, repressed tumour formation in mice, possibly through inhibition of NF-kappaB activation. Futakuchi *et al* (2004), based on results from an *in vivo* metastasis model in rats, suggested that aspirin has the potential to inhibit lung metastasis from chemically induced hepatocarcinoma cells by inhibiting NF-kappaB and its downstream genes.

The results in this thesis extend these reports by examining the effect of meloxicam on human primary tumours. They are consistent with meloxicam inhibiting cell proliferation and inducing apoptosis in oesophageal SCC tissue *in vivo* through mechanisms similar to those by which aspirin and NS398 affected oesophageal SCC TE-13 cells *in vitro*. Together, the *in vitro* and *in vivo* results in Chapters 5 and 6 support further investigation of the potential for aspirin or NSAIDs in oesophageal SCC prevention or treatment.

8.6. Aspirin treatment of patients following oesophagectomy

The results reported in Chapter 4 in this thesis showed a relationship between COX-2 expression and the development and/or outcome of cancer. Subsequent chapters showed that aspirin, NS-398 and meloxicam each inhibited tumour cell proliferation and increased apoptosis either *in vitro* or *in vivo*. These findings were consistent with published studies, reviewed in Chapter 1, which suggested that aspirin or COX-2 inhibitors could reduce the incidence of cancer, including oesophageal.

The long-term survival of patients with cancer of the oesophagus and gastric cardia still remains disappointing, despite extensive research to develop new treatment modalities (Liu et al., 2004). Barbier (1998) reported that 46% of patients who underwent transhiatal oesophagectomy for cancer developed recurrent carcinoma within one year after surgery. While most recurrences were systemic, local recurrence occurred in a significant proportion of the patients. Numerous studies have shown that the process of tumour removal itself appears to stimulate accelerated growth of minimal residual disease, resulting in rapid cancer recurrence at local and systemic levels (Oliver, 1995; Coffey et al., 2003; Qadri et al., 2005a). Preoperative chemotherapy does not inhibit cancer recurrence and spread, and has no survival benefit to patients with SCC of the oesophagus (Chan et al., 2002; Burmeister et al., 2005). Futakuchi *et al* (2004), based on results from an *in vivo* metastasis model in rats, suggested that aspirin has the potential to inhibit lung metastasis from chemically induced hepatocarcinoma cells by inhibiting NF-kappaB and its downstream genes. In a model of recurrent disease, Qadri (2005b) injected tumour cells into the mammary fat pad of mice, then resected the primary growth 2 weeks later. Two weeks after this the mice were killed and the pulmonary metastatic burden assessed. Primary tumour excision was associated with accelerated local and systemic tumour recurrence. However, these effects were significantly attenuated using selective COX-2 inhibition. The COX-2-inhibition was associated with increased levels of apoptosis. Qadri (2005b) concluded that these findings endorsed a role for COX-2 inhibition in the secondary prevention of cancer recurrence at both local and systemic levels.

The aim of the study reported in Chapter 7 was to determine if aspirin would inhibit the development of recurrent cancer in patients after resection of oesophageal tumours, with an improvement in long-term survival. Although the cumulative survival was higher for the patients who took aspirin for at least 12 months after surgery compared to the controls, the increase was not statistically significant. The fact that a similar benefit was seen in both squamous and adenocarcinoma patients makes it hard not to conclude that there may be a subgroup of patients who are benefiting from the aspirin.

This is the first study to investigate the use of aspirin as a post-surgical adjuvant therapy for cancer patients. There are no reports of the use of aspirin alone for the treatment of cancer, although NSAIDs have been reported to enhance the effectiveness of chemotherapy and radiotherapy. Awara (2004) reported that the celecoxib enhanced the anti-tumour activity of doxorubicin in an experimentally-induced mammary tumour, while celecoxib or diclofenac alone did not induce apoptosis in the tumour. Duffy (1998) reported that the addition of a NSAID significantly increased the cytotoxicity of some anticancer drugs *in vitro*, particularly in tumour cells with multidrug resistance-associated protein mediated multidrug resistance. Kobayashi (1997) demonstrated that indomethacin increased the cytotoxicity of vincristine more than a 3-fold in a pulmonary adenocarcinoma cell line *in vitro*. Hida (2002) demonstrated a significant growth inhibition of human lung cancer cells both *in vitro* and *in vivo* by the combined use of a selective COX-2 inhibitor, JTE-522, and conventional anticancer agents. Mizutani showed that combination treatment of bladder cancer cells with the selective COX-2 inhibitor JTE-522 and 5-FU results in synergistic cytotoxicity and apoptosis *in vitro* (Mizutani et al., 2002). The enhancement of radiosensitivity by COX-2 inhibitors has been demonstrated in cancer cells in both *in vivo* and *in vitro* studies (Petersen et al., 2000; Pyo et al., 2001; Raju et al., 2002). The augmentation of conventional therapy by NSAIDs is relevant to the current study, in which chemo-radiotherapy was used for stage IIb or later stage squamous cell carcinoma, and chemotherapy for stage IIb or later stage adenocarcinoma of the gastric cardia.

It is possible that the dose of aspirin selected was not optimal, but there is no reported dose of aspirin for preventing recurrence of cancer *in vivo* in the literature. The dose was selected to minimise the risk of upper gastro-intestinal bleeding. In China the

daily dose of aspirin for the prevention of cerebral and cardiovascular disease is 50 mg. Extensive epidemiological studies have suggested that the duration and continuity of NSAID use may be more critical than daily dose for the prevention of gastrointestinal cancers (Giovannucci et al., 1995; Thun and Heath, 1995; Collet et al., 1999). The mean follow-up in this study was 41 months (the maximum was 67 months) - a longer follow-up may have shown a significant improvement in outcome with aspirin use. Studies involving longer follow-up, different doses of aspirin, or combination therapy, are warranted.

8.7. Conclusions

The aim of the studies described in this thesis was to investigate COX-2 expression in SCC of the oesophagus, and the potential of aspirin or non-steroidal anti-inflammatory drugs, which inhibit the action of the enzyme, for chemoprevention of this cancer. The expression of COX-2 was elevated and correlated with TNM stage and lymph node metastases. Survival was longer in those patients whose tumours expressed lower levels of COX-2. The mechanism of action of aspirin, a non-selective COX inhibitor, and NS-398, a selective COX-2 inhibitor, was investigated *in vitro* in a cell line, and similar changes were seen in tumour tissue resected from patients given the selective COX-2 inhibitor Mobic daily for 14 days before surgery. Although these results suggested that aspirin and similar drugs might have value in cancer therapy, in a clinical trial there was no statistically significant improvement in the survival of patients who were given aspirin for at least 12 months following an oesophagectomy for SCC. However, there was a trend to longer survival in the patients who took the aspirin, suggesting that there should be further studies into its use in this group of patients.

References

Aciyama H, Tsurunmaru M, Udagawa H, Kajiyama Y. (1994), 'Systemic lymph node dissection for oesophageal cancer-effective or not?', *Dis Esoph*, 7:2-14.

Adachi W, Koike S, Nimura Y, Koide N, Iida F, Du XQ, Ping YM, He M, Chen LQ, Zhang MD, Zhang HL. (1996), 'Clinicopathologic characteristics and postoperative outcome in Japanese and Chinese patients with thoracic esophageal cancer', *World J Surg*, 20:332-6.

Ahnen DJ. (1998), 'Colon cancer prevention by NSAIDs: what is the mechanism of action?', *Eur J Surg Suppl*:111-4.

Akiyama H, Tsurumaru M, Udagawa H, Kajiyama Y. (1994), 'Radical lymph node dissection for cancer of the thoracic esophagus', *Ann Surg*, 220:364-72; discussion 372-3.

Alberts DS, Hixson L, Ahnen D, Bogert C, Einspahr J, Paranka N, Brendel K, Gross PH, Pamukcu R, Burt RW. (1995), 'Do NSAIDs exert their colon cancer chemoprevention activities through the inhibition of mucosal prostaglandin synthetase?', *J Cell Biochem Suppl*, 22:18-23.

Alderson D, Courtney SP, Kennedy RH. (1994), 'Radical transhiatal oesophagectomy under direct vision', *Br J Surg*, 81:404-7.

Alexiou C, Watson M, Beggs D, Salama FD, Morgan WE. (1998), 'Chylothorax following oesophagogastrectomy for malignant disease', *Eur J Cardiothorac Sur*, 14:460-6.

Anonymous. (1997), 'Esophageal cancer studies in the Caspian littoral of Iran: results of population studies--a prodrome, Joint Iran-International Agency for Research on Cancer Study Group', *Journal of the National Cancer Institute*, 59:1127-38.

Arico S, Pattingre S, Bauvy C, Gane P, Barbat A, Codogno P, Ogier-Denis E. (2002), 'Celecoxib induces apoptosis by inhibiting 3-phosphoinositide-dependent protein kinase-1 activity in the human colon cancer HT-29 cell line', *J Biol Chem*, 277:27613-21.

Arnott SJ, Duncan W, Kerr GR, Walbaum PR, Cameron E, Jack WJ, Mackillop WJ. (1992), 'Low dose preoperative radiotherapy for carcinoma of the oesophagus: results of a randomized clinical trial', *Radiother Oncol*, 24:108-13.

Attiga FA, Fernandez PM, Weeraratna AT, Manyak MJ, Patierno SR. (2000), 'Inhibitors of prostaglandin synthesis inhibit human prostate tumor cell invasiveness and reduce the release of matrix metalloproteinases', *Cancer Res*, 60:4629-37.

Awara WM, El-Sisi AE, El-Sayad ME, Goda AE. (2004), 'The potential role of cyclooxygenase-2 inhibitors in the treatment of experimentally-induced mammary tumour: does celecoxib enhance the anti-tumour activity of doxorubicin?', *Pharmacol Res*, 50:487-98.

Baek SJ, Kim KS, Nixon JB, Wilson LC, Eling TE. (2001), 'Cyclooxygenase inhibitors regulate the expression of a TGF-beta superfamily member that has proapoptotic and antitumorigenic activities', *Mol Pharmacol*, 59:901-8.

Barbier PA, Becker CD, Wagner HE. (1988), 'Esophageal carcinoma: patient selection for transhiatal esophagectomy, A prospective analysis of 50 consecutive cases', *World J Surg*, 12:263-9.

Barnes CJ, Cameron IL, Hardman WE, Lee M. (1998), 'Non-steroidol anti-inflammatory drug effect on crypt cell proliferation and apoptosis during initiation of rat colon carcinogenesis', *Br J Cancer*, 77:573-80.

Barnes CJ, Hardman WE, Cameron IL, Lee M. (1997), 'Aspirin, but not sodium salicylate, indomethacin, or nabumetone, reversibly suppresses 1,2-dimethylhydrazine-induced colonic aberrant crypt foci in rats', *Dig Dis Sci*, 42:920-6.

Bhansali MS, Vaidya JS, Bhatt RG, Patil PK, Badwe RA, Desai PB. (1996), 'Chemotherapy for carcinoma of the esophagus: a comparison of evidence from meta-analyses of randomized trials and of historical control studies', *Ann Oncol*, 7:355-9.

Blot WJ, Devesa SS, Fraumeni JF Jr. (1993), 'Continuing climb in rates of esophageal adenocarcinoma: an update', *JAMA*, 270:1320.

Blot WJ, Devesa SS, Kneller RW, Fraumeni JF Jr. (1991), 'Rising incidence of adenocarcinoma of the esophagus and gastric cardia', *JAMA*, 265:1287-9.

Bollschweiler E, Schroder W, Holscher AH, Siewert JR. (2000), 'Preoperative risk analysis in patients with adenocarcinoma or squamous cell carcinoma of the oesophagus', *Br J Surg*, 87:1106-10.

Boon EM, Keller JJ, Wormhoudt TA, Giardiello FM, Offerhaus GJ, van der Neut R, Pals ST. (2004), 'Sulindac targets nuclear beta-catenin accumulation and Wnt

signalling in adenomas of patients with familial adenomatous polyposis and in human colorectal cancer cell lines', *Br J Cancer*, 90:224-9.

Bos CL, Kodach LL, van den Brink GR, Diks SH, van Santen MM, Richel DJ, Peppelenbosch MP, Hardwick JC. (2006), 'Effect of aspirin on the Wnt/beta-catenin pathway is mediated via protein phosphatase 2A', *Oncogene,* 25;6447-6456.

Buchanan FG, Wang D, Bargiacchi F, DuBois RN. (2003), 'Prostaglandin E2 regulates cell migration via the intracellular activation of the epidermal growth factor receptor', *J Biol Chem*, 278:35451-7.

Bucher C, Jordan P, Nickeleit V, Torhorst J, Mihatsch MJ. (1999), 'Relative risk of malignant tumors in analgesic abusers, Effects of long-term intake of aspirin', *Clin Nephrol*, 51:67-72.

Burmeister BH, Smithers BM, Gebski V, Fitzgerald L, Simes RJ, Devitt P, Ackland S, Gotley DC, Joseph D, Millar J, North J, Walpole ET, Denham JW. (2005), 'Surgery alone versus chemoradiotherapy followed by surgery for resectable cancer of the oesophagus: a randomised controlled phase III trial', *Lancet Oncol*, 6:659-68.

Chan AC, Lee DW, Griffith JF, Leung SF, Lam YH, Lam CC, Lau JY, Ng EK, Chung SC. (2002), 'The clinical efficacy of neoadjuvant chemotherapy in squamous esophageal cancer: a prospective nonrandomized study of pulse and continuous-infusion regimens with Cisplatin and 5-Fluorouracil', *Ann Surg Oncol*, 9:617-24.

Cheng KK, Day NE, Duffy SW, Lam TH, Fok M, Wong J. (1992), 'Pickled vegetables in the aetiology of oesophageal cancer in Hong Kong Chinese', *Lancet*, 339:1314-8.

Cheong E, Ivory K, Doleman J, Parker ML, Rhodes M, Johnson IT. (2004), 'Synthetic and naturally occurring COX-2 inhibitors suppress proliferation in a human oesophageal adenocarcinoma cell line (OE33) by inducing apoptosis and cell cycle arrest', *Carcinogenesis*, 25:1945-52.

Coffey JC, Wang JH, Smith MJ, Bouchier-Hayes D, Cotter TG, Redmond HP. (2003), 'Excisional surgery for cancer cure: therapy at a cost', *Lancet Oncol*, 4:760-8.

Collet JP, Sharpe C, Belzile E, Boivin JF, Hanley J, Abenhaim L. (1999), 'Colorectal cancer prevention by non-steroidal anti-inflammatory drugs: effects of dosage and timing', *Br J Cancer*, 81:62-8.

Cramer DW, Harlow BL, Titus-Ernstoff L, Bohlke K, Welch WR, Greenberg ER. (1998), 'Over-the-counter analgesics and risk of ovarian cancer', *Lancet*, 351:104-7.

Craven PA and DeRubertis FR. (1992), 'Effects of aspirin on 1,2-dimethylhydrazine-induced colonic carcinogenesis', *Carcinogenesis*, 13:541-6.

Della Bella S, Molteni M, Compasso S, Zulian C, Vanoli M, Scorza R. (1997), 'Differential effects of cyclo-oxygenase pathway metabolites on cytokine production by T lymphocytes', *Prostaglandins Leukot Essent Fatty Acids*, 56:177-84.

DeMeester SR. (2006), 'Adenocarcinoma of the esophagus and cardia: a review of the disease and its treatment', *Ann Surg Oncol*, 13:12-30.

DeMeester TR, Zaninotto G, Johansson KE. (1988), 'Selective therapeutic approach to cancer of the lower esophagus and cardia', *J Thorac Cardiovasc Surg*, 95:42-54.

Dempke W, Rie C, Grothey A, Schmoll HJ. (2001), 'Cyclooxygenase-2: a novel target for cancer chemotherapy?', *J Cancer Res Clin Oncol*, 127:411-7.

Denkert C, Kobel M, Pest S, Koch I, Berger S, Schwabe M, Siegert A, Reles A, Klosterhalfen B, Hauptmann S. (2002), 'Expression of cyclooxygenase 2 is an independent prognostic factor in human ovarian carcinoma', *Am J Pathol*, 160:893-903.

Department of Pathology, Henan Medical College. (1967), 'The use of exfoliative cytology in clinical diagnosis and mass survey for cancer of the esophagus", *Chin Med J*, 6:479. (In Chinese),

Department of Thoracic Surgery, 4th Hospital, Hebei Medical College. (1978), 'Surgical treatment and its long-term results of 3694 patients with cancer of the oesophageu and gastric cardia', *Chung Liu Fang Zhi Yan Jiu (Chin J Cancer Prev Res)*, 1:1-4. (in Chinese)'.

Deutsch ME. (1992), 'Aspirin use and reduced risk of fatal colon cancer', *N Engl J Med*, 326:1289; author reply 1290-1.

Dihlmann S, Klein S, Doeberitz Mv MK. (2003), 'Reduction of beta-catenin/T-cell transcription factor signaling by aspirin and indomethacin is caused by an increased stabilization of phosphorylated beta-catenin', *Mol Cancer Ther*, 2:509-16.

Ding H, Han C, Gibson-D'Ambrosio R, Steele VE, D'Ambrosio SM. (2003),

'Piroxicam selectively inhibits the growth of premalignant and malignant human oral cell lines by limiting their progression through the S phase and reducing the levels of cyclins and AP-1', *Int J Cancer*, 107:830-6.

Ding H, Han C, Zhu J, Chen CS, D'Ambrosio SM. (2005), 'Celecoxib derivatives induce apoptosis via the disruption of mitochondrial membrane potential and activation of caspase 9', *Int J Cancer*, 113:803-10.

Driessen A, Landuyt W, Pastorekova S, Moons J, Goethals L, Haustermans K, Nafteux P, Penninckx F, Geboes K, Lerut T, Ectors N. (2006), 'Expression of carbonic anhydrase IX (CA IX), a hypoxia-related protein, rather than vascular-endothelial growth factor (VEGF), a pro-angiogenic factor, correlates with an extremely poor prognosis in esophageal and gastric adenocarcinomas', *Ann Surg*, 243:334-40.

Du Y, Wabf XL, Wu GX, Liu SZ, Li YQ, Zuo LF. (2004), 'Expression and significance of PCNA and p27 in oesophageal cancer by flowcytometry', *J Clin Exp Pathol,* 20:709-711. （In Chinese）

DuBois RN, Awad J, Morrow J, Roberts LJ 2nd, Bishop PR. (1994), 'Regulation of eicosanoid production and mitogenesis in rat intestinal epithelial cells by transforming growth factor-alpha and phorbol ester', *J Clin Invest*, 93:493-8.

Duffy CP, Elliott CJ, O'Connor RA, Heenan MM, Coyle S, Cleary IM, Kavanagh K, Verhaegen S, O'Loughlin CM, NicAmhlaoibh R, Clynes M. (1998), 'Enhancement of chemotherapeutic drug toxicity to human tumour cells *in vitro* by a subset of non-steroidal anti-inflammatory drugs (NSAIDs)', *Eur J Cancer*, 34:1250-9.

Egan KM, Stampfer MJ, Giovannucci E, Rosner BA, Colditz GA. (1996), 'Prospective study of regular aspirin use and the risk of breast cancer', *J Natl Cancer Inst*, 88:988-93.

Elder DJ, Halton DE, Crew TE, Paraskeva C. (2000), 'Apoptosis induction and cyclooxygenase-2 regulation in human colorectal adenoma and carcinoma cell lines by the cyclooxygenase-2-selective non-steroidal anti-inflammatory drug NS-398', *Int J Cancer*, 86:553-60.

Elder DJ, Halton DE, Hague A, Paraskeva C. (1997), 'Induction of apoptotic cell death in human colorectal carcinoma cell lines by a cyclooxygenase-2 (COX-2)-selective nonsteroidal anti-inflammatory drug: independence from

COX-2 protein expression', *Clin Cancer Res*, 3:1679-83.

Elwood PC. (2001), 'Aspirin: past, present and future', *Clin Med*, 1:132-7.

Elwood PC, Hughes C, O'Brien JR. (1998), 'Platelets, aspirin, and cardiovascular disease', *Postgrad Med J*, 74:587-91.

Engel J and Holzel D. (2001), 'Risk and prognosis of corpus carcinomas after tamoxifen treatment of breast carcinoma', *Strahlenther Onkol*, 177:371.

Farrow DC, Vaughan TL, Hansten PD, Stanford JL, Risch HA, Gammon MD, Chow WH, Dubrow R, Ahsan H, Mayne ST, Schoenberg JB, West AB, Rotterdam H, Fraumeni JF Jr, Blot WJ. (1998), 'Use of aspirin and other nonsteroidal anti-inflammatory drugs and risk of esophageal and gastric cancer', *Cancer Epidemiol Biomarkers Prev*, 7:97-102.

Fietkau R and Kundt G. (2001), 'Problematic results of a randomized study of neoadjuvant radiochemotherapy of operable esophageal carcinoma', *Strahlenther Onkol*, 177:372-3.

Fok M, McShane J, Law SY, Wong J. (1994), 'A prospective randomized study on radiotherapy and surgery in the treatment of esophageal carcinoma', 17:223-229.

Form DM and Auerbach R. (1983), 'PGE2 and angiogenesis', *Proc Soc Exp Biol Med*, 172:214-8.

France M, Drew PA, Dodd T, Watson DI. (2004), 'Cyclo-oxygenase-2 expression in esophageal adenocarcinoma as a determinant of clinical outcome following esophagectomy', *Dis Esophagus*, 17:136-40.

Frantz B and O'Neill EA. (1995), 'The effect of sodium salicylate and aspirin on NF-kappa B', *Science*, 270:2017-9.

Fujita T, Matsui M, Takaku K, Uetake H, Ichikawa W, Taketo MM, Sugihara K. (1998), 'Size- and invasion-dependent increase in cyclooxygenase 2 levels in human colorectal carcinomas', *Cancer Res*, 58:4823-6.

Funkhouser EM and Sharp GB. (1995), 'Aspirin and reduced risk of esophageal carcinoma', *Cancer*, 76:1116-9.

Futakuchi M, Ogawa K, Tamano S, Takahashi S, Shirai T. (2004), 'Suppression of metastasis by nuclear factor kappaB inhibitors in an *in vivo* lung metastasis model of chemically induced hepatocellular carcinoma', *Cancer Sci*, 95:18-24.

Gallois C, Habib A, Tao J, Moulin S, Maclouf J, Mallat A, Lotersztajn S. (1998), 'Role of NF-kappaB in the antiproliferative effect of endothelin-1 and tumor necrosis factor-alpha in human hepatic stellate cells, Involvement of

cyclooxygenase-2', *J Biol Chem*, 273:23183-90.

Gann PH, Manson JE, Glynn RJ, Buring JE, Hennekens CH. (1993), 'Low-dose aspirin and incidence of colorectal tumors in a randomized trial', *J Natl Cancer Inst*, 85:1220-4.

Gaut ZN. (1993), 'Aspirin use and risk of fatal cancer, Cancer Res, 53: 1322-1327, 1993', *Cancer Res*, 53:6074.

Geh JI, Crellin AM, Glynne-Jones R. (2001), 'Preoperative (neoadjuvant) chemoradiotherapy in oesophageal cancer', *Br J Surg*, 88:338-56.

Ghadirian P. (1987), 'Thermal irritation and esophageal cancer in northern Iran', *Cancer*, 60:1909-14.

Giardina C, Boulares H, Inan MS. (1999), 'NSAIDs and butyrate sensitize a human colorectal cancer cell line to TNF-alpha and Fas ligation: the role of reactive oxygen species', *Biochim Biophys Acta*, 1448:425-38.

Gignoux M, Roussel A, Paillot B, Gillet M, Schlag P, Favre JP, Dalesio O, Buyse M, Duez N. (1987), 'The value of preoperative radiotherapy in esophageal cancer: results of a study of the E.O.R.T.C', *World J Surg*, 11:426-32.

Giovannucci E, Egan KM, Hunter DJ, Stampfer MJ, Colditz GA, Willett WC, Speizer FE. (1995), 'Aspirin and the risk of colorectal cancer in women', *N Engl J Med*, 333:609-14.

Giovannucci E, Rimm EB, Stampfer MJ, Colditz GA, Ascherio A, Willett WC. (1994), 'Aspirin use and the risk for colorectal cancer and adenoma in male health professionals', *Ann Intern Med*, 121:241-6.

Goldberg Y, Nassif II, Pittas A, Tsai LL, Dynlacht BD, Rigas B, Shiff SJ. (1996), 'The anti-proliferative effect of sulindac and sulindac sulfide on HT-29 colon cancer cells: alterations in tumor suppressor and cell cycle-regulatory proteins', *Oncogene*, 12:893-901.

Goodnight SH. (1996), 'Aspirin therapy for cardiovascular disease', *Curr Opin Hematol*, 3:355-60.

Goodwin JS and Ceuppens J. (1983), 'Regulation of the immune response by prostaglandins', *J Clin Immunol*, 3:295-315.

Greer SE, Goodney PP, Sutton JE, Birkmeyer JD. (2005), 'Neoadjuvant chemoradiotherapy for esophageal carcinoma: a meta-analysis', *Surgery*, 137:172-7.

Grosch S, Tegeder I, Niederberger E, Brautigam L, Geisslinger G. (2001), 'COX-2

independent induction of cell cycle arrest and apoptosis in colon cancer cells by the selective COX-2 inhibitor celecoxib', *FASEB J*, 15:2742-4.

Gupta RA, Tan J, Krause WF, Geraci MW, Willson TM, Dey SK, DuBois RN. (2000), 'Prostacyclin-mediated activation of peroxisome proliferator-activated receptor delta in colorectal cancer', *Proc Natl Acad Sci U S A*, 97:13275-80.

Hanahan D and Folkman J. (1996), 'Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis', *Cell*, 86:353-64.

Hanif R, Pittas A, Feng Y, Koutsos MI, Qiao L, Staiano-Coico L, Shiff SI, Rigas B. (1996), 'Effects of nonsteroidal anti-inflammatory drugs on proliferation and on induction of apoptosis in colon cancer cells by a prostaglandin-independent pathway', *Biochem Pharmacol*, 52:237-45.

Hansen-Petrik MB, McEntee MF, Jull B, Shi H, Zemel MB, Whelan J. (2002), 'Prostaglandin E_2 protects intestinal tumors from nonsteroidal anti-inflammatory drug-induced regression in Apc (Min/+) mice', *Cancer Res*, 62:403-8.

Harris SG, Padilla J, Koumas L, Ray D, Phipps RP. (2002), 'Prostaglandins as modulators of immunity', *Trends Immunol*, 23:144-50.

Hashemolhosseini S, Nagamine Y, Morley SJ, Desrivieres S, Mercep L, Ferrari S. (1998), 'Rapamycin inhibition of the G1 to S transition is mediated by effects on cyclin D1 mRNA and protein stability', *J Biol Chem*, 273:14424-9.

He TC, Chan TA, Vogelstein B, Kinzler KW. (1999), 'PPARdelta is an APC-regulated target of nonsteroidal anti-inflammatory drugs', *Cell*, 99:335-45.

Hebei Provincial Office of Cancer Prevention and Treatment. (1980), 'Data on cancer prevention and reasearch', 4:2. (In Chinese),

Heeren P, Plukker J, van Dullemen H, Nap R, Hollema H. (2005), 'Prognostic role of cyclooxygenase-2 expression in esophageal carcinoma', *Cancer Lett*, 225:283-9.

Hempel SL, Monick MM, Hunninghake GW. (1994), 'Lipopolysaccharide induces prostaglandin H synthase-2 protein and mRNA in human alveolar macrophages and blood monocytes', *J Clin Invest*, 93:391-6.

Hida T, Kozaki K, Ito H, Miyaishi O, Tatematsu Y, Suzuki T, Matsuo K, Sugiura T, Ogawa M, Takahashi T, Takahashi T. (2002), 'Significant growth inhibition of human lung cancer cells both *in vitro* and *in vivo* by the combined use of a selective cyclooxygenase 2 inhibitor, JTE-522, and conventional anticancer agents', *Clin Cancer Res*, 8:2443-7.

Hida T, Yatabe Y, Achiwa H, Muramatsu H, Kozaki K, Nakamura S, Ogawa M,

Mitsudomi T, Sugiura T, Takahashi T. (1998), 'Increased expression of cyclooxygenase 2 occurs frequently in human lung cancers, specifically in adenocarcinomas', *Cancer Res*, 58:3761-4.

Holash J, Maisonpierre PC, Compton D, Boland P, Alexander CR, Zagzag D, Yancopoulos GD, Wiegand SJ. (1999), 'Vessel cooption, regression, and growth in tumors mediated by angiopoietins and VEGF', *Science*, 284:1994-8.

Holla VR, Wang D, Brown JR, Mann JR, Katkuri S, DuBois RN. (2005), 'Prostaglandin E2 regulates the complement inhibitor CD55/decay-accelerating factor in colorectal cancer', *J Biol Chem*, 280:476-83.

Honjo S, Kase S, Osaki M, Ardyanto TD, Kaibara N, Ito H. (2005), 'COX-2 correlates with F-box protein, Skp2 expression and prognosis in human gastric carcinoma', *Int J Oncol*, 26:353-60.

Hou J, Chen ZF, Zhang GS, et al. (1996), 'Study on blocking progress of precancerous lesions of the esophagus by Cang Dou Wan', *Chin Oncol Clin*, 17:17-19. (In Chinese)

Hou J, Lin PS, Chen ZF. (1998), 'A study survey of oesophageal cancer in Cixian of Hebei', *Chung Liu Fang Zhi Yan Jiu*, 25:73-75.

Hou J, Zhang H, Liu TG, He YT, Yang SQ, Ma Z. (1995), 'Analysis of cancer mortality in 21 country in Hebei from year 1990 through 1992', *Hebei Medicine (Hebei Yiyao),* 17:20-38. (In Chinese),

Hsu AL, Ching TT, Wang DS, Song X, Rangnekar VM, Chen CS. (2000), 'The cyclooxygenase-2 inhibitor celecoxib induces apoptosis by blocking Akt activation in human prostate cancer cells independently of Bcl-2', *J Biol Chem*, 275:11397-403.

Huang GJ. (1991), Surgical treatment for cancer of the oesophagus and gastric cardia, In: *Criteria of Diagnosis and Treatment of Commonly Encountered Malignant Tumours in China*, Beijing:Joint publishing house of Beijing Medical University and Union Medical University, 29-31.

Huang M, Stolina M, Sharma S, Mao JT, Zhu L, Miller PW, Wollman J, Herschman H, Dubinett SM. (1998), 'Non-small cell lung cancer cyclooxygenase-2-dependent regulation of cytokine balance in lymphocytes and macrophages: up-regulation of interleukin 10 and down-regulation of interleukin 12 production', *Cancer Res*, 58:1208-16.

Husain SS, Szabo IL, Tamawski AS. (2002), 'NSAID inhibition of GI cancer growth:

clinical implications and molecular mechanisms of action', *Am J Gastroenterol*, 97:542-53.

Hwang D, Scollard D, Byrne J, Levine E. (1998), 'Expression of cyclooxygenase-1 and cyclooxygenase-2 in human breast cancer', *J Natl Cancer Inst*, 90:455-60.

Imdahl A, Bognar G, Schulte-Monting J, Schoffel U, Farthmann EH, Ihling C. (2002), 'Predictive factors for response to neoadjuvant therapy in patients with oesophageal cancer', *Eur J Cardiothorac Surg*, 21:657-63.

Jaskiewicz K, Marasas WF, van der Walt FE. (1987), 'Oesophageal and other main cancer patterns in four districts of Transkei, 1981-1984', *S Afr Med J*, 72:27-30.

Jiang JG, Tang JB, Chen CL, Liu BX, Fu XN, Zhu ZH, Qu W, Cianflone K, Waalkes MP, Wang DW. (2004), 'Expression of cyclooxygenase-2 in human esophageal squamous cell carcinomas', *World J Gastroenterol*, 10:2168-73.

Jones MK, Wang H, Peskar BM, Levin E, Itani RM, Sarfeh IJ, Tarnawski AS. (1999), 'Inhibition of angiogenesis by nonsteroidal anti-inflammatory drugs: insight into mechanisms and implications for cancer growth and ulcer healing', *Nat Med*, 5:1418-23.

Jones RG, Parsons M, Bonnard M, Chan VS, Yeh WC, Woodgett JR, Ohashi PS. (2000), 'Protein kinase B regulates T lymphocyte survival, nuclear factor kappaB activation, and Bcl-X (L) levels *in vivo*', *J Exp Med*, 191:1721-34.

Kandil HM, Tanner G, Smalley W, Halter S, Radhika A, Dubois RN. (2001), 'Cyclooxygenase-2 expression in Barrett's esophagus', *Dig Dis Sci*, 46:785-9.

Kang HJ, Song MJ, Choung SY, Kim SJ, Le MO. (2000), 'Transcriptional induction of Nur77 by indomethacin that results in apoptosis of colon cancer cells', *Biol Pharm Bull*, 23:815-9.

Kang S, Kim YB, Kim MH, Yoon KS, Kim JW, Park NH, Song YS, Kang D, Yoo KY, Kang SB, Lee HP. (2005), 'Polymorphism in the nuclear factor kappa-B binding promoter region of cyclooxygenase-2 is associated with an increased risk of bladder cancer', *Cancer Lett*, 217:11-6.

Kashfi K and Rigas B. (2005), 'Non-COX-2 targets and cancer: expanding the molecular target repertoire of chemoprevention', *Biochem Pharmacol*, 70:969-86.

Kauppi M, Pukkala E, Isomaki H. (1996), 'Low incidence of colorectal cancer in patients with rheumatoid arthritis', *Clin Exp Rheumatol*, 14:551-3.

Kaur BS, Khamnehei N, Iravani M, Namburu SS, Lin O, Triadafilopoulos G. (2002),

'Rofecoxib inhibits cyclooxygenase 2 expression and activity and reduces cell proliferation in Barrett's esophagus', *Gastroenterology*, 123:60-7.

Kawamori T, Rao CV, Seibert K, Reddy BS. (1998), 'Chemopreventive activity of celecoxib, a specific cyclooxygenase-2 inhibitor, against colon carcinogenesis', *Cancer Res*, 58:409-12.

Kawamori T, Uchiya N, Sugimura T, Wakabayashi K. (2003), 'Enhancement of colon carcinogenesis by prostaglandin E2 administration', *Carcinogenesis*, 24:985-90.

Kivela AJ, Kivela J, Saarnio J, Parkkila S. (2005), 'Carbonic anhydrases in normal gastrointestinal tract and gastrointestinal tumours', *World J Gastroenterol*, 11:155-63.

Kmet J and Mahboubi E. (1972), 'Esophageal cancer in the Caspian littoral of Iran: initial studies', *Science*, 175:846-53.

Kobayashi S, Okada S, Yoshida H, Fujimura S. (1997), 'Indomethacin enhances the cytotoxicity of VCR and ADR in human pulmonary adenocarcinoma cells', *Tohoku J Exp Med*, 181:361-70.

Koga H, Sakisaka S, Ohishi M, Kawaguchi T, Taniguchi E, Sasatomi K, Harada M, Kusaba T, Tanaka M, Kimura R, Nakashima Y, Nakashima O, Kojiro M, Kurohiji T, Sata M. (1999), 'Expression of cyclooxygenase-2 in human hepatocellular carcinoma: relevance to tumor dedifferentiation', *Hepatology*, 29:688-96.

Kojima M, Morisaki T, Izuhara K, Uchiyama A, Matsunari Y, Katano M, Tanaka M. (2000), 'Lipopolysaccharide increases cyclo-oxygenase-2 expression in a colon carcinoma cell line through nuclear factor-kappa B activation', *Oncogene*, 19:1225-31.

Kuang AA, Cado D, Winoto A. (1999), 'Nur77 transcription activity correlates with its apoptotic function *in vivo*', *Eur J Immunol*, 29:3722-8.

Kune GA, Kune S, Watson LF. (1988), 'Colorectal cancer risk, chronic illnesses, operations, and medications: case control results from the Melbourne Colorectal Cancer Study', *Cancer Res*, 48:4399-404.

Kuo KT, Chow KC, Wu YC, Lin CS, Wang HW, Li WY, Wang LS. (2003), 'Clinicopathologic significance of cyclooxygenase-2 overexpression in esophageal squamous cell carcinoma', *Ann Thorac Surg*, 76:909-14.

Langman MJ, Cheng KK, Gilman EA, Lancashire RJ. (2000), 'Effect of anti-inflammatory drugs on overall risk of common cancer: case-control study in

general practice research database', *BMJ*, 320:1642-6.

Launois B, Delarue D, Campion JP, Kerbaol M. (1981), 'Preoperative radiotherapy for carcinoma of the esophagus', *Surg Gynecol Obstet*, 153:690-2.

Law BK, Waltner-Law ME, Entingh AJ, Chytil A, Aakre ME, Norgaard P, Moses HL. (2000), 'Salicylate-induced growth arrest is associated with inhibition of p70s6k and down-regulation of c-myc, cyclin D1, cyclin A, and proliferating cell nuclear antigen', *J Biol Chem*, 275:38261-7.

Law, SWJ. (1999), 'Does lymphadenectomy add anything to the treatment of esophageal cancer?', *Advances in Surgery*, 311-27.

Law, SWJ. (2001), 'What is appropriate treatment for carcinoma of the thoracic esophagus?', *World Journal of Surgery*, 25:189-95.

Lee R and Collins T, (2001), 'Nuclear factor-kappaB and cell survival: IAPs call for support', *Circ Res*, 88:262-4.

Levy GN. (1997), 'Prostaglandin H synthases, nonsteroidal anti-inflammatory drugs, and colon cancer', *FASEB J*, 11:234-47.

Li H, Schut HA, Conran P, Kramer PM, Lubet RA, Steele VE, Hawk EE, Kelloff GJ, Pereira MA. (1999), 'Prevention by aspirin and its combination with alpha-difluoromethylornithine of azoxymethane-induced tumors, aberrant crypt foci and prostaglandin E2 levels in rat colon', *Carcinogenesis*, 20:425-30.

Li H and Yao SC. (1997), 'Surgical treatment for carcinoma of the oesophagus in Chinese language publications', *Br J Sur*, 84:855-7.

Li JY. (1982), 'Epidemiology of esophageal cancer in China', *Natl Cancer Inst Monogr*, 62:113-20.

Li LD, Lu FZ, Zhang SW, Mu R, Sun XD, Huangpu XM, Sun J, Zhou YS, Ouyang NH, Rao KQ, Chen YD, Sun AM, Xue ZF, Xia Y. (1996), 'Analysis of cancer mortality and distribution in China from year 1990 through 1992, An epidemiological study', *Chin J Oncol*, 18:403-407. (in Chinese）.

Li M, Lotan R, Levin B, Tahara E, Lippman SM, Xu XC. (2000), 'Aspirin induction of apoptosis in esophageal cancer: a potential for chemoprevention', *Cancer Epidemiol Biomarkers Prev*, 9:545-9.

Li M, Wu X, Xu XC. (2001), 'Induction of apoptosis by cyclo-oxygenase-2 inhibitor NS398 through a cytochrome C-dependent pathway in esophageal cancer cells', *Int J Cancer*, 93:218-23.

Li MH, Li P, Li PJ. (1980), 'Recent progress in research on esophageal cancer in China', *Adv Cancer Res*, 33:173-249.

Li Y, Li Y, Tang R, Xu H, Qiu M, Chen Q, Chen J, Fu Z, Ying K, Xie Y, Mao Y. (2002), 'Discovery and analysis of hepatocellular carcinoma genes using cDNA microarrays', *J Cancer Res Clin Oncol*, 128:369-79.

Lim JW, Kim H, Kim KH. (2001), 'Nuclear factor-kappaB regulates cyclooxygenase-2 expression and cell proliferation in human gastric cancer cells', *Lab Invest*, 81:349-60.

Liu JF, Wang QZ, Hou J. (2004), 'Surgical treatment for cancer of the oesophagus and gastric cardia in Hebei, China', *Br J Surg*, 91:90-8.

Liu JF, Wang QZ, Li BQ, Meng XL, Zhang YD, Du XQ, Yan JS, Pin YM, Li WQ. (1995), 'Superficial carcinomas of the esophagus and gastric cardia, A clinicopathological analysis of 141 cases', *Chin Med J*, 108:754-759.

Liu JF, Zhang HL, Li R. (1996), 'A comparative study of operation only and operation with postoperative irradiation in esophageal cancer', *Pract J Cancer*, 11:1-3. (In Chinese),

Lu D, Cottam HB, Corr M, Carson DA. (2005), 'Repression of beta-catenin function in malignant cells by nonsteroidal antiinflammatory drugs', *Proc Natl Acad Sci U S A*, 102:18567-71.

Lu SH, Camus AM, Tomatis L, Bartsch H. (1981), 'Mutagenicity of extracts of pickled vegetables collected in Linhsien County, a high-incidence area for esophageal cancer in Northern China', *J Natl Cancer Inst*, 66:33-6.

Luo Y, Yoshizawa T, Katayama T. (1990), 'Comparative study on the natural occurrence of Fusarium mycotoxins (trichothecenes and zearalenone) in corn and wheat from high- and low-risk areas for human esophageal cancer in China', *Appl Environ Microbiol*, 56:3723-6.

Lupulescu A. (1996), 'Prostaglandins, their inhibitors and cancer', *Prostaglandins Leukot Essent Fatty Acids*, 54:83-94.

Ma Z, Hou J, Ling H, et al. (1989), 'Sampled research on reasons of deaths in Hebei Province', *Hebei medicine (Hebei Yiyao)*: (supple):2. (In Chinese),

Maaser K, Daubler P, Barthel B, Heine B, von Lampe B, Stein H, Hoffmeister B, Scherer H, Scherubl H. (2003), 'Oesophageal squamous cell neoplasia in head and neck cancer patients: upregulation of COX-2 during carcinogenesis', *Br J Cancer*, 88:1217-22.

Mahboubi E, Kmet J, Cook PJ, Day NE, Ghadirian P, Salmasizadeh S. (1973), 'Oesophageal cancer studies in the Caspian Littoral of Iran: the Caspian cancer registry', *Br J Cancer*, 28:197-214.

Manson JE, Stampfer MJ, Colditz GA, Willett WC, Rosner B, Speizer FE, Hennekens CH. (1991), 'A prospective study of aspirin use and primary prevention of cardiovascular disease in women', *JAMA*, 266:521-7.

Marnett LJ. (1992), 'Aspirin and the potential role of prostaglandins in colon cancer', *Cancer Res*, 52:5575-89.

Marnett LJ. (1999), 'Lipid peroxidation-DNA damage by malondialdehyde', *Mutat Res*, 424:83-95.

Marnett LJ, Basu AK, O'Hara SM, Weller PE, Rahman AFMM, and Oliver JP. (1986), 'Reaction of malondialdehyde with guanine nucleosides: formation of adducts containing oxadrazabicyclononene residues in the base-pairing region', *J Am Chem Soc*, 108:1348–1350.

Marx J. (2001), 'Cancer research, Anti-inflammatories inhibit cancer growth--but how?', *Science*, 291:581-2.

Mascaux C, Martin B, Paesmans M, Berghmans T, Dusart M, Haller A, Lothaire P, Meert AP, Lafitte JJ, Sculier JP. (2006), 'Has Cox-2 a prognostic role in non-small-cell lung cancer? A systematic review of the literature with meta-analysis of the survival results', *Br J Cancer*, 95:139-45.

Masferrer JL, Isakson PC, Seibert K. (1996), 'Cyclooxygenase-2 inhibitors: a new class of anti-inflammatory agents that spare the gastrointestinal tract', *Gastroenterol Clin North Am*, 25:363-72.

Masferrer JL, Leahy KM, Koki AT, Zweifel BS, Settle SL, Woerner BM, Edwards DA, Flickinger AG, Moore RJ, Seibert K. (2000), 'Antiangiogenic and antitumor activities of cyclooxygenase-2 inhibitors', *Cancer Res*, 60:1306-11.

McDougall CJ, Ngoi SS, Goldman IS, Godwin T, Felix J, DeCosse JJ, Rigas B. (1990), 'Reduced expression of HLA class I and II antigens in colon cancer', *Cancer Res,* 50:8023-7.

Merigliano S, Molena D, Ruol A, Zaninotto G, Cagol M, Scappin S, Ancona E. (2000), 'Chylothorax complicating esophagectomy for cancer: a plea for early thoracic duct ligation', *J Thorac Cardiovasc Surg*, 119:453-7.

Miller BA, Ries LAG, Hankey BF, Kosary CL, Edwards BK, eds. (1992), *Cancer Statistics Review: 1973-1989*. Bethesda: National Cancer Institute.

Mizutani Y, Kamoi K, Ukimura O, Kawauchi A, Miki T. (2002), 'Synergistic cytotoxicity and apoptosis of JTE-522, a selective cyclooxygenase-2 inhibitor, and 5-fluorouracil against bladder cancer', *J Urol*, 168:2650-4.

Morris CD, Armstrong GR, Bigley G, Green H, Attwood SE. (2001), 'Cyclooxygenase-2 expression in the Barrett's metaplasia-dysplasia-adenocarcinoma sequence', *Am J Gastroenterol*, 96:990-6.

Muir CS, Waterhouse J, Mack T, Powell J, Whelan S. (1987), *Cancer incidence in 5 countries*.

Murono S, Yoshizaki T, Sato H, Takeshita H, Furukawa M, Pagano JS. (2000), 'Aspirin inhibits tumor cell invasiveness induced by Epstein-Barr virus latent membrane protein 1 through suppression of matrix metalloproteinase-9 expression', *Cancer Res*, 60:2555-61.

Nishihira T, Hirayama K, Mori S. (1998), 'A prospective randomized trial of extended cervical and superior mediastinal lymphadenectomy for carcinoma of the thoracic esophagus', *Am J Surg*, 175:47-51.

Nozoe T, Ezaki T, Kabashima A, Baba H, Maehara Y. (2005), 'Significance of immunohistochemical expression of cyclooxygenase-2 in squamous cell carcinoma of the esophagus', *Am J Surg*, 189:110-5.

Nygaard K, Hagen S, Hansen HS, Hatlevoll R, Hultborn R, Jakobsen A, Mantyla M, Modig H, Munck-Wikland E, Rosengren B, et al. (1992), 'Pre-operative radiotherapy prolongs survival in operable esophageal carcinoma: a randomized, multicenter study of pre-operative radiotherapy and chemotherapy, The second Scandinavian trial in esophageal cancer', *World J Surg*, 16:1104-9.

Okami J, Yamamoto H, Fujiwara Y, Tsujie M, Kondo M, Noura S, Oshima S, Nagano H, Dono K, Umeshita K, Ishikawa O, Sakon M, Matsuura N, Nakamori S, Monden M. (1999), 'Overexpression of cyclooxygenase-2 in carcinoma of the pancreas', *Clin Cancer Res*, 5:2018-24.

Oliver RT. (1995), 'Does surgery disseminate or accelerate cancer?', *Lancet*, 346:1506-7.

O'Neill C, Jordan P, Bhatt T and Newman R. (1986), 'Silica and esophageal cancer', *Ciba Foundation Symposium*, 121:214-30.

Orringer MB, Marshall B, Iannettoni MD. (1999), 'Transhiatal esophagectomy: clinical experience and refinements', *Ann Surg*, 230:392-400; discussion 400-3.

Oshima M, Dinchuk JE, Kargman SL, Oshima H, Hancock B, Kwong E, Trzaskos JM,

Evans JF, Taketo MM. (1996), 'Suppression of intestinal polyposis in Apc delta716 knockout mice by inhibition of cyclooxygenase 2 (COX-2)', *Cell*, 87:803-9.

Paganini-Hill A. (1995), 'Aspirin and colorectal cancer: the Leisure World cohort revisited', *Prev Med*, 24:113-5.

Paganini-Hill A, Hsu G, Ross RK, Henderson BE. (1991), 'Aspirin use and incidence of large-bowel cancer in a California retirement community', *J Natl Cancer Inst*, 83:1182-3.

Pai R, Soreghan B, Szabo IL, Pavelka M, Baatar D, Tarnawski AS. (2002), 'Prostaglandin E2 transactivates EGF receptor: a novel mechanism for promoting colon cancer growth and gastrointestinal hypertrophy', *Nat Med*, 8:289-93.

Parkin DM, Pisani P, Ferlay J. (1999), 'Estimates of the worldwide incidence of 25 major cancers in 1990', *Int J Cancer*, 80:827-41.

Parkin DM, Pisani P, Ferlay J. (1993), 'Estimates of the worldwide incidence of eighteen major cancers in 1985', *Int J Cancer*, 54:594-606.

Peleg II, Maibach HT, Brown SH, Wilcox CM. (1994), 'Aspirin and nonsteroidal anti-inflammatory drug use and the risk of subsequent colorectal cancer', *Arch Intern Med*, 154:394-9.

Pera M, Cameron AJ, Trastek VF, Carpenter HA, Zinsmeister AR. (1993), 'Increasing incidence of adenocarcinoma of the esophagus and esophagogastric junction', *Gastroenterology*, 104:510-3.

Pera M, Manterola C, Vidal O, Grande L. (2005), 'Epidemiology of esophageal adenocarcinoma', *J Surg Oncol*, 92:151-9.

Petersen C, Petersen S, Milas L, Lang FF, Tofilon PJ. (2000), 'Enhancement of intrinsic tumor cell radiosensitivity induced by a selective cyclooxygenase-2 inhibitor', *Clin Cancer Res*, 6:2513-20.

Piazza GA, Alberts DS, Hixson LJ, Paranka NS, Li H, Finn T, Bogert C, Guillen JM, Brendel K, Gross PH, Sperl G, Ritchie J, Burt RW, Ellsworth L, Ahnen DJ, Pamukcu R. (1997), 'Sulindac sulfone inhibits azoxymethane-induced colon carcinogenesis in rats without reducing prostaglandin levels', *Cancer Res*, 57:2909-15.

Pierce JW, Read MA, Ding H, Luscinskas FW, Collins T. (1996), 'Salicylates inhibit I kappa B-alpha phosphorylation, endothelial-leukocyte adhesion molecule expression, and neutrophil transmigration', *J Immunol*, 156:3961-9.

Ping YM. (2002), 'The development of surgical treatment for cancer of the esophagus and gastric cardia', *Hebei Provincial Symposium on esophageal cancer and begnine diseases*, ShijiazhuangShijiazhuang.

Ping YM, He M, Chen LQ, Yan JS. (1995), 'Analysis on causes of postoperative deaths following resection of cancer of the esophagus and gastric cardia', *Proceedings of Fourth China National Symposium on Esophageal Cancer,* Nanjing, 1995;13'.

Ping YM, Yan JS, Du XQ, Wang DZ, Zhang YD. (1982), 'Anastomotic leakage after oesophagectomy and oesophagogastrostomy for cancer of the oesophagus and gastric cardia, An analysis of 159 cases', *Chin J Oncol*, 4:36-38.

Ping YM, Zhang YD, Zhang W, Wang ZL, Du XQ. (1983), 'Diaphragmatic hernia after resection of carcinoma of the esophagus and gastric cardia', *Advances in Medicine*, 4:250-253.

Pinotti HW, Cecconello I, De Oliveira MA. (1997), 'Transhiatal esophagectomy for esophageal cancer', *Semin Surg Oncol*, 13:253-8.

Pisani P, Parkin DM, Ferlay J. (1993), 'Estimates of the worldwide mortality from eighteen major cancers in 1985, Implications for prevention and projections of future burden', *Int J Cancer*, 55:891-903.

Poligone B and Baldwin AS. (2001), 'Positive and negative regulation of NF-kappaB by COX-2: roles of different prostaglandins', *J Biol Chem*, 276:38658-64.

Pottern LM, Morris LE, Blot WJ, Ziegler RG, Fraumeni JF Jr. (1981), 'Esophageal cancer among black men in Washington, D.C, I, Alcohol, tobacco, and other risk factors', *J Natl Cancer Inst*, 67:777-83.

Prescott SM and White RL. (1996), 'Self-promotion? Intimate connections between APC and prostaglandin H synthase-2', *Cell*, 87:783-6.

Puscas I, Coltau M, Pasca R. (1996), 'Nonsteroidal anti-inflammatory drugs activate carbonic anhydrase by a direct mechanism of action', *J Pharmacol Exp Ther*, 277:1464-6.

Pyo H, Choy H, Amorino GP, Kim JS, Cao Q, Hercules SK, DuBois RN. (2001), 'A selective cyclooxygenase-2 inhibitor, NS-398, enhances the effect of radiation *in vitro* and *in vivo* preferentially on the cells that express cyclooxygenase-2', *Clin Cancer Res*, 7:2998-3005.

Qadri SS, Wang JH, Coffey JC, Alam M, O'Donnell A, Aherne T, Redmond HP. (2005a), 'Can surgery for cancer accelerate the progression of secondary tumors

within residual minimal disease at both local and systemic levels?', *Ann Thorac Surg*, 80:1046-50.

Qadri SS, Wang JH, Coffey JC, Alam M, O'Donnell A, Aherne T, Redmond HP. (2005b), 'Surgically induced accelerated local and distant tumor growth is significantly attenuated by selective COX-2 inhibition', *Ann Thorac Surg*, 79:990-5.

Qiao L, Shiff SJ, Rigas B. (1997), 'Sulindac sulfide inhibits the proliferation of colon cancer cells: diminished expression of the proliferation markers PCNA and Ki-67', *Cancer Lett*, 115:229-34.

Raju U, Nakata E, Yang P, Newman RA, Ang KK, Milas L. (2002), '*In vitro* enhancement of tumor cell radiosensitivity by a selective inhibitor of cyclooxygenase-2 enzyme: mechanistic considerations', *Int J Radiat Oncol Biol Phys*, 54:886-94.

Ratnasinghe D, Tangrea J, Roth MJ, Dawsey S, Hu N, Anver M, Wang QH, Taylor PR. (1999), 'Expression of cyclooxygenase-2 in human squamous cell carcinoma of the esophagus; an immunohistochemical survey', *Anticancer Res*, 19:171-4.

Reddy BS, Rao CV, Rivenson A, Kelloff G. (1993), 'Inhibitory effect of aspirin on azoxymethane-induced colon carcinogenesis in F344 rats', *Carcinogenesis*, 14:1493-7.

Reeves MJ, Newcomb PA, Trentham-Dietz A, Storer BE, Remington PL. (1996), 'Nonsteroidal anti-inflammatory drug use and protection against colorectal cancer in women', *Cancer Epidemiol Biomarkers Prev*, 5:955-60.

Rigas B, Goldman IS, Levine L. (1993), 'Altered eicosanoid levels in human colon cancer', *J Lab Clin Med*, 122:518-23.

Rigas B, Tsioulias GJ, Allan C, Wali RK, Brasitus TA. (1994), 'The effect of bile acids and piroxicam on MHC antigen expression in rat colonocytes during colon cancer development', *Immunology*, 83:319-23.

Rindani R, Martin CJ, Cox MR. (1999), 'Transhiatal versus Ivor-Lewis oesophagectomy: is there a difference?', *Aust N Z J Surg*, 69:187-94.

Ristimaki A, Honkanen N, Jankala H, Sipponen P, Harkonen M. (1997), 'Expression of cyclooxygenase-2 in human gastric carcinoma', *Cancer Res*, 57:1276-80.

Ristimaki A, Sivula A, Lundin J, Lundin M, Salminen T, Haglund C, Joensuu H, Isola J. (2002), 'Prognostic significance of elevated cyclooxygenase-2 expression in breast cancer', *Cancer Res*, 62:632-5.

Rolland PH, Martin PM, Jacquemier J, Rolland AM, Toga M. (1980), 'Prostaglandin in human breast cancer: Evidence suggesting that an elevated prostaglandin production is a marker of high metastatic potential for neoplastic cells', *J Natl Cancer Inst*, 64:1061-70.

Rosenberg L. (1996), 'Aspirin and breast cancer: no surprises yet', *J Natl Cancer Inst*, 88:941-2.

Rosenberg L, Louik C, Shapiro S. (1998), 'Nonsteroidal antiinflammatory drug use and reduced risk of large bowel carcinoma', *Cancer*, 82:2326-33.

Rosenberg L, Palmer JR, Zauber AG, Warshauer ME, Stolley PD, Shapiro S. (1991), 'A hypothesis: nonsteroidal anti-inflammatory drugs reduce the incidence of large-bowel cancer', *J Natl Cancer Inst*, 83:355-8.

Rubio CA. (1984), 'Antitumoral activity of indomethacin on experimental esophageal tumors', *J Natl Cancer Inst*, 72:705-7.

Rubio CA. (1986), 'Further studies on the therapeutic effect of indomethacin on esophageal tumors', *Cancer*, 58:1029-31.

Sandler RS, Galanko JC, Murray SC, Helm JF, Woosley JT. (1998), 'Aspirin and nonsteroidal anti-inflammatory agents and risk for colorectal adenomas', *Gastroenterology*, 114:441-7.

Sano H, Kawahito Y, Wilder RL, Hashiramoto A, Mukai S, Asai K, Kimura S, Kato H, Kondo M, Hla T. (1995), 'Expression of cyclooxygenase-1 and -2 in human colorectal cancer', *Cancer Res*, 55:3785-9.

Schreinemachers DM and Everson RB. (1994), 'Aspirin use and lung, colon, and breast cancer incidence in a prospective study', *Epidemiology*, 5:138-46.

Sclabas GM, Uwagawa T, Schmidt C, Hess KR, Evans DB, Abbruzzese JL, Chiao PJ. (2005), 'Nuclear factor kappa B activation is a potential target for preventing pancreatic carcinoma by aspirin', *Cancer*, 103:2485-90.

Shamma A, Yamamoto H, Doki Y, Okami J, Kondo M, Fujiwara Y, Yano M, Inoue M, Matsuura N, Shiozaki H, Monden M. (2000), 'Up-regulation of cyclooxygenase-2 in squamous carcinogenesis of the esophagus', *Clin Cancer Res*, 6:1229-38.

Shao J, Fujiwara T, Kadowaki Y, Fukazawa T, Waku T, Itoshima T, Yamatsuji T, Nishizaki M, Roth JA, Tanaka N. (2000), 'Overexpression of the wild-type p53 gene inhibits NF-kappaB activity and synergizes with aspirin to induce apoptosis in human colon cancer cells', *Oncogene*, 19:726-36.

Shao, L.F. (2002), 'Esophageal cancer', In: Shao LF, Wang QZ eds, *Surgery of the esophagus*. Hebei Publishing House of Science and Technology, Shijiazhuang:

Sharma RA. (2002), 'Translational medicine: targetting cyclo-oxygenase isozymes to prevent cancer', *QJM*, 95:267-73.

Shen Q and Wang GQ. (1998), 'Esophageal Cancer', *Cytologic screening for carcinoma and dysplasia of the esophagus in the People's Republic of China*:25-31.

Sheng H, Shao J, Morrow JD, Beauchamp RD, DuBois RN. (1998), 'Modulation of apoptosis and Bcl-2 expression by prostaglandin E2 in human colon cancer cells', *Cancer Res*, 58:362-6.

Sheng H, Shao J, Washington MK, DuBois RN. (2001), 'Prostaglandin E2 increases growth and motility of colorectal carcinoma cells', *J Biol Chem*, 276:18075-81.

Shiff SJ, Koutsos MI, Qiao L, Rigas B. (1996), 'Nonsteroidal antiinflammatory drugs inhibit the proliferation of colon adenocarcinoma cells: effects on cell cycle and apoptosis', *Exp Cell Res*, 222:179-88.

Shiff SJ, Qiao L, Tsai LL, Rigas B. (1995), 'Sulindac sulfide, an aspirin-like compound, inhibits proliferation, causes cell cycle quiescence, and induces apoptosis in HT-29 colon adenocarcinoma cells', *J Clin Invest*, 96:491-503.

Shiff SJ and Rigas B. (1997), 'Nonsteroidal anti-inflammatory drugs and colorectal cancer: evolving concepts of their chemopreventive actions', *Gastroenterology*, 113:1992-8.

Shirahama T, Arima J, Akiba S, Sakakura C. (2001), 'Relation between cyclooxygenase-2 expression and tumor invasiveness and patient survival in transitional cell carcinoma of the urinary bladder', *Cancer*, 92:188-93.

Shirvani VN, Ouatu-Lascar R, Kaur BS, Omary MB, Triadafilopoulos G. (2000), 'Cyclooxygenase 2 expression in Barrett's esophagus and adenocarcinoma: Ex vivo induction by bile salts and acid exposure', *Gastroenterology*, 118:487-96.

Shreedhar V, Giese T, Sung VW, Ullrich SE. (1998), 'A cytokine cascade including prostaglandin E2, IL-4, and IL-10 is responsible for UV-induced systemic immune suppression', *J Immunol*, 160:3783-9.

Siebenlist U, Franzoso G, Brown K. (1994), 'Structure, regulation and function of NF-kappa B', *Annu Rev Cell Biol*, 10:405-55.

Siewert JR and Stein HJ. (1999), 'Lymph-node dissection in squamous cell esophageal cancer -- who benefits?', *Langenbecks Arch Surg*, 384:141-8.

Sivula A, Buskens CJ, van Rees BP, Haglund C, Offerhaus GJ, van Lanschot JJ, Ristimaki A. (2005), 'Prognostic role of cyclooxygenase-2 in neoadjuvant-treated patients with squamous cell carcinoma of the esophagus', *Int J Cancer*, 116:903-8.

Slater MS, Holland J, Faigel DO, Sheppard BC, Deveney CW. (2001), 'Does neoadjuvant chemoradiation downstage esophageal carcinoma?', *Am J Surg*, 181:440-4.

Smalley W, Ray WA, Daugherty J, Griffin MR. (1999), 'Use of nonsteroidal anti-inflammatory drugs and incidence of colorectal cancer: a population-based study', *Arch Intern Med*, 159:161-6.

Song S and Xu XC. (2001), 'Effect of benzo[a]pyrene diol epoxide on expression of retinoic acid receptor-beta in immortalized esophageal epithelial cells and esophageal cancer cells', *Biochem Biophys Res Commun*, 281:872-7.

Sovak MA, Bellas RE, Kim DW, Zanieski GJ, Rogers AE, Traish AM, Sonenshein GE. (1997), 'Aberrant nuclear factor-kappaB/Rel expression and the pathogenesis of breast cancer', *J Clin Invest*, 100:2952-60.

Stevenson MA, Zhao MJ, Asea A, Coleman CN, Calderwood SK. (1999), 'Salicylic acid and aspirin inhibit the activity of RSK2 kinase and repress RSK2-dependent transcription of cyclic AMP response element binding protein- and NF-kappa B-responsive genes', *J Immunol*, 163:5608-16.

Sturmer T, Glynn RJ, Lee IM, Manson JE, Buring JE, Hennekens CH. (1998), 'Aspirin use and colorectal cancer: post-trial follow-up data from the Physicians' Health Study', *Ann Intern Med*, 128:713-20.

Suh O, Mettlin C, Petrelli NJ. (1993), 'Aspirin use, cancer, and polyps of the large bowel', *Cancer*, 72:1171-7.

Tegeder I, Pfeilschifter J, Geisslinger G. (2001), 'Cyclooxygenase-independent actions of cyclooxygenase inhibitors', *FASEB J*, 15:2057-72.

Thun MJ and Heath CW. (1995), 'Aspirin use and reduced risk of gastrointestinal tract cancers in the American Cancer Society prospective studies', *Prev Med*, 24:116-8.

Thun MJ, Namboodiri MM, Calle EE, Flanders WD, Heath CW Jr. (1993), 'Aspirin use and risk of fatal cancer', *Cancer Res*, 53:1322-7.

Thun MJ, Namboodiri MM, Heath CW Jr. (1991), 'Aspirin use and reduced risk of fatal colon cancer', *N Engl J Med*, 325:1593-6.

Tsioulias G, Godwin TA, Goldstein MF, McDougall CJ, Ngoi SS, DeCosse JJ, Rigas B. (1992), 'Loss of colonic HLA antigens in familial adenomatous polyposis', *Cancer Res JT - Cancer research*, 52:3449-52.

Tsioulias GJ, Triadafilopoulos G, Goldin E, Papavassiliou ED, Rizos S, Bassioukas P, Rigas B. (1993), 'Expression of HLA class I antigens in sporadic adenomas and histologically normal mucosa of the colon', *Cancer Res*, 53:2374-8.

Tsui SL, Law S, Fok M, Lo JR, Ho E, Yang J, Wong J. (1997), 'Postoperative analgesia reduces mortality and morbidity after esophagectomy', *Am J Surg*, 173:472-8.

Tsujii M, Kawano S, Tsuji S, Sawaoka H, Hori M, DuBois RN. (1998), 'Cyclooxygenase regulates angiogenesis induced by colon cancer cells', *Cell*, 93:705-16.

Tucker ON, Dannenberg AJ, Yang EK, Zhang F, Teng L, Daly JM, Soslow RA, Masferrer JL, Woerner BM, Koki AT, Fahey TJ 3rd. (1999), 'Cyclooxygenase-2 expression is up-regulated in human pancreatic cancer', *Cancer Res*, 59:987-90.

Tuyns AJ, (1982), 'Cancer of the esophagus' in:, PfeifferCJ eds, *Epidemiology of esophageal cancer in French*, 3-18.

Uefuji K, Ichikura T, Mochizuki H. (2000), 'Cyclooxygenase-2 expression is related to prostaglandin biosynthesis and angiogenesis in human gastric cancer', *Clin Cancer Res*, 6:135-8.

Urschel JD, Vasan H, Blewett CJ. (2002), 'A meta-analysis of randomized controlled trials that compared neoadjuvant chemotherapy and surgery to surgery alone for resectable esophageal cancer', *Am J Surg*, 183:274-9.

van den Brink MR, Kapeller R, Pratt JC, Chang JH, Burakoff SJ. (1999), 'The extracellular signal-regulated kinase pathway is required for activation-induced cell death of T cells', *J Biol Chem*, 274:11178-85.

van Rensburg SJ. (1981), 'Epidemiologic and dietary evidence for a specific nutritional predisposition to esophageal cancer', *J Natl Cancer Inst*, 67:243-51.

van Rensburg SJ. (1987), 'Oesophageal cancer risk factors common to endemic regions', *S Afr Med J*, Suppl:9-11.

Vane JR. (1971), 'Inhibition of prostaglandin synthesis as a mechanism of action for aspirin-like drugs', *Nat New Biol*, 231:232-5.

Vane SJ. (2000), 'Aspirin and other anti-inflammatory drugs', *Thorax*, 55 Suppl 2:S3-9.

Visbal AL, Allen MS, Miller DL, Deschamps C, Trastek VF, Pairolero PC. (2001), 'Ivor Lewis esophagogastrectomy for esophageal cancer', *Ann Thorac Surg*, 71:1803-8.

Wang CY, Mayo MW, Korneluk RG, Goeddel DV, Baldwin AS Jr. (1998), 'NF-kappaB antiapoptosis: induction of TRAF1 and TRAF2 and c-IAP1 and c-IAP2 to suppress caspase-8 activation', *Science*, 281:1680-3.

Wang D, Buchanan FG, Wang H, Dey SK, DuBois RN. (2005), 'Prostaglandin E2 enhances intestinal adenoma growth via activation of the Ras-mitogen-activated protein kinase cascade', *Cancer Res*, 65:1822-9.

Wang D, Wang H, Shi Q, Katkuri S, Walhi W, Desvergne B, Das SK, Dey SK, DuBois RN. (2004), 'Prostaglandin E (2) promotes colorectal adenoma growth via transactivation of the nuclear peroxisome proliferator-activated receptor delta', *Cancer Cell*, 6:285-95.

Wang M, Gu XZ, Yin WB, Huang GJ, Wang LJ, Zhang DW. (1989), 'Randomized clinical trial on the combination of preoperative irradiation and surgery in the treatment of esophageal carcinoma: report on 206 patients', *Int J Radiat Oncol Biol Phys*, 16:325-7.

Wang QZ, Du XQ, Zhang YD. (1985), 'Prevention and treatment of chylothorax after surgery for esophageal cancer', *Acta Academiae Medicine Hebei*, 6:95-97.

Wang YP, Han XY, Su W, Wang YL, Zhu YW, Sasaba T, Nakachi K, Hoshiyama Y, Tagashira Y. (1992), 'Esophageal cancer in Shanxi Province, People's Republic of China: a case-control study in high and moderate risk areas', *Cancer Causes Control*, 3:107-13.

Watson A and Allen PR. (1994), 'Influence of thoracic epidural analgesia on outcome after resection for esophageal cancer', *Surgery*, 115:429-32.

Whooley BP, Law S, Alexandrou A, Murthy SC, Wong J. (2001), 'Critical appraisal of the significance of intrathoracic anastomotic leakage after esophagectomy for cancer', *Am J Surg*, 181:198-203.

Williams CS, Tsujii M, Reese J, Dey SK, DuBois RN. (2000), 'Host cyclooxygenase-2 modulates carcinoma growth', *J Clin Invest*, 105:1589-94.

Wilson AJ, Arango D, Mariadason JM, Heerdt BG, Augenlicht LH. (2003), 'TR3/Nur77 in colon cancer cell apoptosis', *Cancer Res*, 63:5401-7.

Wilson KT, Fu S, Ramanujam KS, Meltzer SJ. (1998), 'Increased expression of inducible nitric oxide synthase and cyclooxygenase-2 in Barrett's esophagus and

associated adenocarcinomas', *Cancer Res*, 58:2929-34.

Wolff H, Saukkonen K, Anttila S, Karjalainen A, Vainio H, Ristimaki A. (1998), 'Expression of cyclooxygenase-2 in human lung carcinoma', *Cancer Res*, 58:4997-5001.

Wong BC, Jiang XH, Lin MC, Tu SP, Cui JT, Jiang SH, Wong WM, Yuen MF, Lam SK, Kung HF. (2004), 'Cyclooxygenase-2 inhibitor (SC-236) suppresses activator protein-1 through c-Jun NH2-terminal kinase', *Gastroenterology*, 126:136-47.

Wulczyn FG, Krappmann D, Scheidereit C. (1996), 'The NF-kappa B/Rel and I kappa B gene families: mediators of immune response and inflammation', *J Mol Med*, 74:749-69.

Xu XC. (2002), 'COX-2 inhibitors in cancer treatment and prevention, a recent development', *Anticancer Drugs*, 13:127-37.

Yamamoto Y and Gaynor RB. (2001), 'Therapeutic potential of inhibition of the NF-kappaB pathway in the treatment of inflammation and cancer', *J Clin Invest*, 107:135-42.

Yang CS. (1980), 'Research on esophageal cancer in China: a review', *Cancer Res*, 40:2633-44.

Yang CS, Sun Y, Yang QU, Miller KW, Li GY, Zheng SF, Ershow AG, Blot WJ, Li JY. (1984), 'Vitamin A and other deficiencies in Linxian, a high esophageal cancer incidence area in northern China', *J Natl Cancer Inst*, 73:1449-53.

Yang L, Yamagata N, Yadav R, Brandon S, Courtney RL, Morrow JD, Shyr Y, Boothby M, Joyce S, Carbone DP, Breyer RM. (2003), 'Cancer-associated immunodeficiency and dendritic cell abnormalities mediated by the prostaglandin EP2 receptor', *J Clin Invest*, 111:727-35.

Yin MJ, Yamamoto Y, Gaynor RB. (1998), 'The anti-inflammatory agents aspirin and salicylate inhibit the activity of I (kappa)B kinase-beta', *Nature*, 396:77-80.

Youn HD, Sun L, Prywes R, Liu JO. (1999), 'Apoptosis of T cells mediated by Ca2+-induced release of the transcription factor MEF2', *Science*, 286:790-3.

Yu HP, Shi LY, Lu WH, Su YH, Li YY, Xu SQ. (2004), 'Expression of cyclooxygenase-2 (COX-2) in human esophageal cancer and *in vitro* inhibition by a specific COX-2 inhibitor, NS-398', *J Gastroenterol Hepatol*, 19:638-42.

Yu HP, Xu SQ, Liu L, Shi LY, Cai XK, Lu WH, Lu B, Su YH, Li YY. (2003), 'Cyclooxygenase-2 expression in squamous dysplasia and squamous cell

carcinoma of the esophagus', *Cancer Lett*, 198:193-201.

Zaridze DG, Basieva T, Kabulov M, Day NE, Duffy SW. (1992), 'Oesophageal cancer in the Republic of Karakalpakstan', *Int J Epidemiol*, 21:643-8.

Zhang DW, Cheng GY, Huang GJ, Zhang RG, Liu XY, Mao YS, Wang YG, Chen SJ, Zhang LZ, Wang LJ, Zhang DC, Yang L, Meng PJ, Sun KL. (1994), 'Operable squamous esophageal cancer: current results from the East', *World J Surg*, 18:347-54.

Zhang F, Subbaramaiah K, Altorki N, Dannenberg AJ. (1998), 'Dihydroxy bile acids activate the transcription of cyclooxygenase-2', *J Biol Chem*, 273:2424-8.

Zhang HL, Meng XL, Ping YM, Wang QZ, Bai SX. (1993), 'Intrathoracic and intraabdominal bleeding after oesophagectomy for cancer- a report of 22 cases', *Acta Academiae Medicine Hebei*, 14:21-23.

Zhang HL, Ping YM, Du XQ, Bai SX, He QB. (1999), 'Study on factors influencing survival in patients with cancer of the esophagus after resection by Cox proportional hazard model', *Chin J Oncol*, 21:32-34. (In Chinese),

Zhang W, Wang L, Chang A, Jin Y, Rao J. (2003), 'Immunohistochemical analysis of cyclooxygenase-2 expression in premalignant and malignant esophageal glandular and squamous lesions in Cixian, China', *Cancer Detect Prev*, 27:243-9.

Zhang X, Miao X, Tan W, Ning B, Liu Z, Hong Y, Song W, Guo Y, Zhang X, Shen Y, Qiang B, Kadlubar FF, Lin D. (2005), 'Identification of functional genetic variants in cyclooxygenase-2 and their association with risk of esophageal cancer', *Gastroenterology*, 129:565-76.

Zhang YD,Du XQ, Yang JS, Zhang W, Ping YM, Ma WH, Zhang X, Guo BZ, Wan J. (1998), 'Treatment of carcinoma in the middle third of the oesophagus using preoperative Cobalt^{60} radiation combined with surgery (comparative study analysis of 200 cases)', *J Thorac Cardiovasc Surg,* 4: 30-33. (In Chinese)

Zhang YD, Du XQ, Zhang MG, Yan JS, Wang QZ, Ping YM,Wang SZ, Zhang MD, Li WQ, Song YC, Bai SX, Li BQ, Wang ZL, Chen Y, Wang FS, Li FL, Ma YQ, Jin LW, Fan ZL. (1989), 'Surgical treatment and long-term outcomes of cancer of the esophagus and gastric cardia', *Proceedings of 2nd China National Symposium on Esophageal Cancer, Shijiazhuang*, 33: (In Chinese),

Zhang YD, Du XQ, Zhang W, Yan JS, Wang QZ, Zhang MG. (1982), 'The experience in surgical treatment of 4310 patients with cancer of esophagus and gastric cardia', *Acta Academiae Medicine Hebei*, 4:1-7. (In Chinese),

Zhang YD, Du XQ, Zhang W, Zhang MG, Yan JS, Wang QZ, Wang DZ, Shi SR, Ping YM, Li WQ, Song YC. (1984), 'Surgical treatment of cancer of the esophagus and gastric cardia', *Acta Academiae Medicine Hebei*, 5:21-22. (In Chinese),

Zhi H, Zhang J, Hu G, Lu J, Wang X, Zhou C, Wu M, Liu Z. (2003), 'The deregulation of arachidonic acid metabolism-related genes in human esophageal squamous cell carcinoma', *Int J Cancer*, 106:327-33.

Ziegler RG, Morris LE, Blot WJ, Pottern LM, Hoover R, Fraumeni JF Jr. (1981), 'Esophageal cancer among black men in Washington, D.C, II, Role of nutrition', *J Natl Cancer Inst*, 67:1199-206.

Zimmermann KC, Sarbia M, Weber AA, Borchard F, Gabbert HE, Schror K. (1999), 'Cyclooxygenase-2 expression in human esophageal carcinoma', *Cancer Res*, 59:198-204.

Zuo LF, Liu HX, Guo JW, Yang ZB, Lin YZ. (1987), 'Preparation of single cell suspensiong from paraffin embeded cancer tissues for flow cytometric analysis', *Chin J Clin Exp Pathol*, 3:239-240. (In Chinese)

Zuo LF. (1988), 'Flow cytometry of breast carcinoma', *Chin J Pathol,* 17:42-45. (In Chinese)