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Can Expression of CXCL12 and CXCR4 Be Used to Predict Survival of Gastric Cancer Patients?

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Can Expression of CXCL12 and CXCR4 Be Used to Predict Survival of Gastric Cancer Patients?

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Abstract. *Background: Currently, there is no effective therapy for advanced gastric cancer. In this study, we investigated whether protein expression of CXCL12 and/or its receptor CXCR4 is associated with clinicopathological features and/or survival of gastric cancer. Materials and Methods: Primary tumor specimens from patients (n=137) with pathologically-confirmed gastric cancer, collected between 2001 and 2009, were analyzed by immunohistochemistry using anti-CXCL12 and anti-CXCR4 antibodies. Results: Expression of CXCL12 was directly associated with tumor differentiation (p=0.0143) but inversely associated with depth of invasion (p=0.0255), lymphatic invasion (p=0.0173), venous invasion (p=0.0022) and stage (p=0.049). Expression of CXCR4 was associated with depth of invasion (p=0.005) and stage (p=0.028). Increased CXCR4 expression, but not CXCL12 expression, was associated with 5-year cancer-specific survival (p=0.0079). Conclusion: CXCL12 was not associated with survival. Positive CXCR4 expression in gastric carcinoma was significantly associated with poor survival and, therefore, may be a potential biomarker for predicting poor survival.*

Gastric cancer is a common disease and remains a major health concern in the Far East as well as in other countries worldwide (1). Prognosis of early-stage gastric cancer is excellent and various minimally-invasive procedures have been exploited to dissect and cure these lesions (2). However, patients with advanced gastric cancer have a poor prognosis and although various treatments have been developed, currently there is no effective therapy available. Therefore, biomarkers that are more precise are required to predict prognosis or aid in the pathological diagnosis.

At present, the properties of chemokines, including trafficking of leukocytes during immune and inflammatory reactions and organ selective migration of cancer cells during metastasis, have been widely recognized. Chemokines are a superfamily of small peptide molecules involved in a number of physiological processes. In humans, this superfamily includes more than 46 ligands and 18 chemokine receptors (3). Under normal physiological conditions, chemokines play a role in both pro-inflammatory and non-inflammatory cell homing. Chemokines are 8-10 kDa cytokines that are classified into four groups (CXC, CC, C and CX3C). Chemokine receptors belong to the G-protein-coupled receptor superfamily. Chemokines/chemokine receptors regulate a variety of immune responses to infection, inflammation and tissue repair. Besides controlling the trafficking of immune cells, chemokines also regulate the migration of seven different cell types during embryogenesis. Recently, the chemokine CXCL12, also known as stromal cell-derived factor-1 (SDF-1), and its receptor CXCR4 were shown to have prominent roles in the initiation and progression of primary and metastatic breast, pancreatic, colorectal, melanoma, neuroblastoma, esophageal, prostate, kidney and lung cancers (4-12).

The aims of this retrospective study were to examine the relationship between CXCL12 and CXCR4 expression and their immunostaining patterns, as well as to explore the role of these proteins, along with clinicopathological factors, play in gastric cancer-specific survival using surgical specimens from gastric cancer patients.

Materials and Methods

Patient characteristics and tissue samples. Our study included 137 patients who were diagnosed with gastric cancer in the Department of Surgery I, Dokkyo Medical University Hospital, between 2001 and 2009. The cohort comprised of 102 males and 35 females with a mean age of 68.9 years (range, 42-93 years) who had all undergone surgical resection. Patients who had

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Key Words: CXCL12, CXCR4, gastric carcinoma, immunohistochemistry.

distant metastasis or peritoneal dissemination were excluded from the study. Surgical diagnosis of residual tumor in these patients was R0 and R1.

Clinical follow-up data, including overall survival, was available for all patients. Median follow-up time of the surviving patients at the time of analysis was 79 months. The pathological tumor stage was classified according to the TNM Classification of Malignant Tumors, 7th Edition. Clinicopathological findings were assessed according to criteria established by the Japanese General Rules for Gastric Cancer Study (13, 14). Written informed consent for pathology analysis of surgical samples was obtained from each patient in accordance with our institution's guidelines prior to enrollment.

Surgical specimens were fixed in 10% formalin, embedded in paraffin and cut into 5-μm thick sections that were then stained with hematoxylin and eosin, and evaluated microscopically by two pathologists.

Immunohistochemistry (IHC). Immunohistochemical staining of CXCL12 and CXCR4 was performed as described previously (15). In brief, the 5-μm thick sections were de-paraffinized, rehydrated, placed in 0.01 mol/l citrate buffer (pH 6.0) and heated in a microwave oven for 20 min. Sections were then preincubated with 3% H_2O_2 in methanol for 30 min at room temperature to quench endogenous peroxidase activity. Sections were pretreated with 1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) and then incubated with an anti-CXCL12 antibody (R&M Systems Inc., Minneapolis, MN, USA; Catalog# MA350: dilution 1:50) and an anti-CXCR4 antibody (Epitomics Inc., Burlingame, CA, USA; Catalog# 3108-1; dilution 1:200) for 1 h at room temperature. Thereafter, sections were incubated with a biotinylated secondary antibody (Vectastain Elite ABC kit, Burlingame, CA, USA) for 30 min, washed with PBS and treated with peroxidase-conjugated streptavidin for 20 min. Finally, sections were visualized by incubating them with diaminobenzidine tetrahydrochloride (Liquid DAB + Substrate Chromogen System; Dako, Carpinteria, CA, USA). Sections were rinsed briefly in water, counterstained with hematoxylin and mounted.

Evaluation of immunohistochemical staining. Two independent investigators blinded to patients' clinicopathological data evaluated the immunohistochemical staining for CXCL12 and CXCR4. Positive expression was defined as the presence of CXCL12 and CXCR4 immunoreactivity in >30% of cancer cells (16).

Due to the heterogeneous content of proliferative tumor cells in sections, areas of highest proliferative activity were found by scanning tumor sections at low magnification. Tumor cell counts were then performed on these areas at a × cell magnification. CXCR4 immunoreactivity was detected in the plasma membrane, cytoplasm and nucleus. Some cancer cells showed clear CXCR4 immunoreactivity in the nucleus and a weak signal in the cytoplasm (nuclear type), whereas others showed no nuclear immunoreactivity but rather a diffuse signal in the cytoplasm and the plasma membrane (cytomembrane type). Every lesion was classified as a nuclear or cytomembrane type in accordance with its dominant immunostaining pattern. However, sections with no CXCR4 immunoreactivity were defined as negative (12).

Statistical analysis. The χ^2 test was performed to determine correlations among the various parameters while Fischer's exact test was also used, as needed. The cumulative survival rate was assessed using the Kaplan-Meier method and analyzed using the log-rank test. Survival curves were estimated using the Kaplan-Meier product-limit method and the significance of difference between survival curves was determined using the log-rank test. A *p*-value of less than 0.05 was considered statistically significant. All statistical analyses were conducted using Stat View 5.0 (SAS Institute Inc., Cary, NC, USA).

Results

Patient demographics and disease characteristics. The study cohort consisted of 137 patients, out of which 102 were males and 35 were females, with a median age of 68.9 years. Based on histological typing, 88 cases (64.2%) were differentiated adenocarcinomas and 49 cases (35.8%) were undifferentiated adenocarcinomas. Thirty-six cases were stage I, 30 cases were stage II, 53 cases were stage III and 18 cases were stage IV (non-curative resection). During the follow-up period, 45 patients died due to relapse and metastasis while 17 patients died due to other causes.

Expression of CXCL12 and CXCR4 in primary tumor tissues. Among the 137 cases of primary gastric cancer, 43 cases (31.4%) stained positive for CXCL12 (Figure 1A, 1B). All positive CXCL12 immunoreactivity was of the cytomembrane type. In addition, 82 cases (59.8%) stained positive for CXCR4 (Figure 1C, 1D). CXCR4 immunoreactivity was of the cytomembrane type in 42 cases and of the nuclear type in 40 cases (Figure 2).

Correlation of CXCL12 and CXCR4 expression with clinicopathological disease characteristics and cancer-specific survival. To explore the clinicopathological significance of CXCL12 and CXCR4 expression, we studied its relationship with several clinicopathological factors. Tables I and II show the correlation of CXCL12 and CXCR4 expression with each clinical feature. Positive expression of CXCL12 in cancer cells was associated with differentiation (p=0.0143), negative depth of invasion (p=0.0255), negative lymphatic invasion (p=0.0173), negative venous invasion (p=0.0022) and early stage (p=0.049). Positive expression of CXCR4 in cancer cells was associated with depth of invasion (p=0.005) and stage (p=0.028). However, there was no correlation between the immunoreactivity pattern and any clinicopathological parameter in either group (Table III). With regard to patient cancer-specific survival, gastric cancer with positive expression of CXCL12 was not associated with a lower 5-year probability of survival compared to those with negative expression of CXCL12 (p=0.9088). Gastric cancer with positive expression of CXCR4, however, was associated with a lower 5-year probability of survival than those with negative expression of CXCR4 (p=0.0079) (Figure 3). There was no correlation between the immunoreactivity pattern and cancer-specific survival (Figure 4).

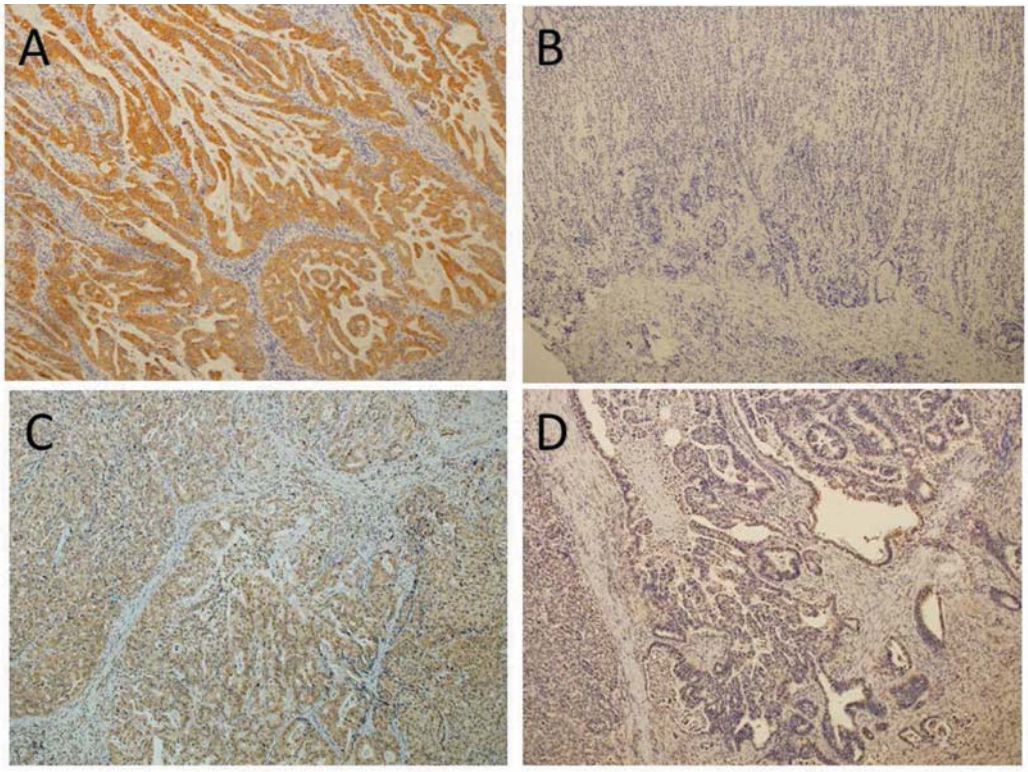

Figure 1. *Representative images of immunostaining of CXCL12 and CXCR4 proteins in gastric cancer tissues. Tumor cells with: (A) Positive and (B) negative expression of CXCL12; and (C) positive and (D) negative expression of CXCR4. Original magnification, ×100.*

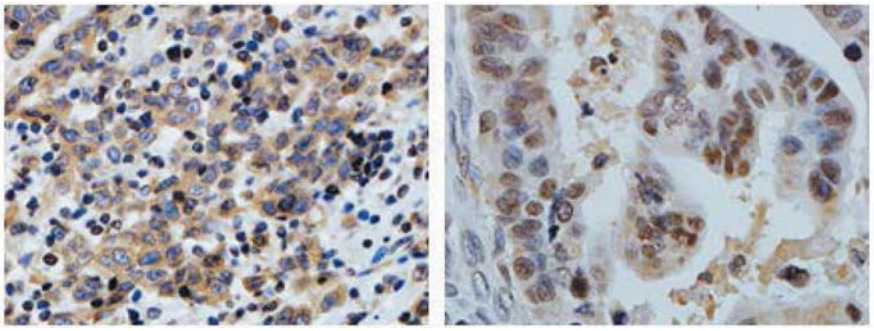

Figure 2. *Immunohistochemistry of CXCR4 in gastric cancer. (A) Cytomembrane type: immunoreactivity was detected in the cytoplasm and plasma membrane of cancer cells. (B) Nuclear type: immunoreactivity was detected weakly in the cytoplasm of cancer cells and strongly in the nucleus. Original magnification, ×1,000.*

Discussion

In 2001, Muller *et al*. published the first report stating that CXCR4 and CCR were highly expressed in human breast cancer tissues and cell lines as well as lymph nodes, lung, liver and bone marrow. These areas have been identified as common metastatic sites of primary breast cancer and highly express CXCL12 (CXCR4 ligand) and CCL21 (CCR7 ligand) (4).

We examined the expression of the chemokine receptor CXCR4 and its ligand CXCL12 in gastric cancer tissues using IHC and analyzed the relationship between their expression and clinicopathological features. CXCL12 was significantly expressed in gastric adenocarcinomas of the intestinal type rather than the diffuse type. In addition, CXCL12 positive expression was inversely associated with depth of invasion, lymphatic invasion and venous invasion.

Table I. *Correlation of CXCL12 expression in tumor cells with clinicopathological patient characteristics.*

Parameters	CXCL12 (+) N=43	CXCL12 (–) N=94	*p*-value
Gender			
Male	35	67	0.207
Female	8	27	
Differentiation			
Differentiated	34	54	0.0143
Undifferentiated	9	40	
Depth of invasion			
T1,2	17	20	0.0255
T3,4	26	74	
Regional lymph nodes			
(–)	18	31	
(+)	25	63	0.314
Lymphatic invasion			
Negative	14	14	0.0173
Positive	29	80	
Venous invasion			
Negative	19	18	0.0022
Positive	24	76	
Stage			
I	16	20	0.049
II, III, IV	27	74	

Table II. *Correlation of CXCR4 expression in tumor cells with clinicopathological patient characteristics.*

Parameters	CXCR4 (+) N=82	CXCR4 (–) N=55	*p*-Value
Gender			
Male	63	39	0.436
Female	19	16	
Differentiation			
Differentiated	50	38	0.331
Undifferentiated	32	17	
Depth of invasion			
T1,2	15	22	0.005
T3,4	67	33	
Regional lymph nodes			
(–)	26	23	0.226
(+)	56	32	
Lymphatic invasion			
Negative	14	14	0.233
Positive	68	41	
Venous invasion			
Negative	20	17	0.399
Positive	62	38	
Stage			
I	16	20	0.028
II, III, IV	66	35	

Increased expression occurred in early-stage gastric cancer, a trend that decreased with gastric tumor progression. Ishigami *et al*. (17) showed that CXCL12 positive expression in gastric cancer was associated with lymph node metastasis, depth of invasion, lymphatic invasion, tumor diameter, clinical stage and poor surgical outcome. Conversely, Ingold *et al*. (18) found that CXCL12 expression was negatively associated with distant metastasis and tumor grade, while other clinicopathological factors were not significantly associated.

CXCL12 is a ubiquitous α-chemokine expressed by a variety of cells and considered a constitutive chemokine (19). Shibata *et al*. demonstrated that overexpression of CXCL12 in the stomach caused a significant enhancement of gastric epithelial cell proliferation, induction of inflammation and tumor development, particularly in combination with *Helicobacter felis* infection (20). Moreover, Fakhari *et al*. reported that co-culture of *H. pylori* with a gastric epithelial cell line, AGS, produced an increase of CXCL12 in AGS cells (21). In our study, *H. pylori* infection was not examined. However, we surmise that carcinogenesis of gastric cancer can occur in a chronic inflammatory environment.

During the early invasive stage, the tumor maintains its epithelial character. During the advanced invasive stage, the epithelial character is lost; therefore, positive expression of CXCL12 is downregulated during the advanced stages of gastric cancer. Lee *et al*. (22) reported that CXCL12 stimulated CXCR4 expression in gastric cancer through both autocrine and paracrine mechanisms. Thus, an autocrine mechanism may attenuate the secretion of CXCL12 helping to accelerate tumor invasion. However, in a previous report, CXCL12 in gastric cancer was not detected in the early stage but was upregulated in advanced invasive tumors. Although further studies are needed, CXCL12 positive expression did not correlate with patient survival in the current study. Therefore, CXCL12 may not be important as a predictive factor.

CXCR4 protein expression was associated with depth of invasion, stage and a lower 5-year probability of cancer-specific survival status after radical resection. Kwak *et al*. (23) detected the expression of *CXCR4* mRNA in 10 gastric cancer cell lines and in 100% (43/43) of gastric cancer tissues using reverse transcription polymerase chain reaction (RT-PCR). This group also reported positive expression of CXCR4 protein in 36.5% of gastric cancer tissues using IHC. Arigami *et al*. (16) demonstrated that CXCR4 expression was related to gastric tumor metastasis to lymph nodes.

Jieer *et al*. (24) detected positive staining of CXCL12 and CXCR4 in gastric cancer cells and metastatic lymph nodes. Moreover, positive staining for CXCR4 was significantly

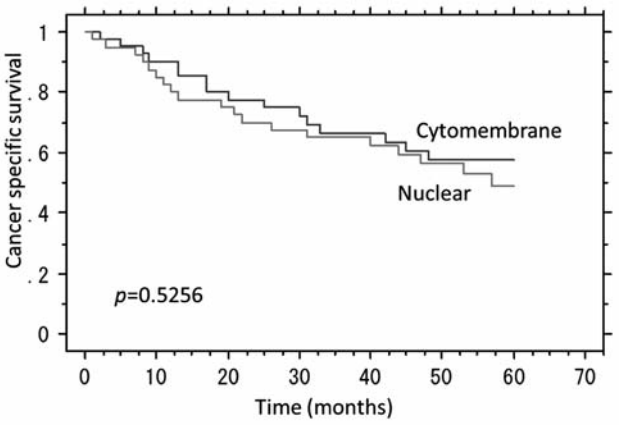

Figure 3. *Kaplan-Meier plot of cancer-specific survival based on cytomembrane-versus nuclear-type expression of CXCR4 in gastric tumor specimens.*

Table III. *Relationship between CXCR4 immunostaining patterns and clinicopathological features in patients with CXCR4-positive gastric tumors.*

Parameters	Nuclear N=40	Cytomembrane N=42	*p*-Value
Gender			
Male	32	31	0.507
Female	8	11	
Depth of invasion			
T1,2	7	8	0.856
T3,4	33	34	
Regional lymph nodes			
(–)	11	15	0.424
(+)	29	27	
Lymphatic invasion			
Negative	8	6	0.492
Positive	32	36	
Venous invasion			
Negative	10	10	0.900
Positive	30	32	
Stage			
I	8	8	0.913
II, III, IV	32	34	

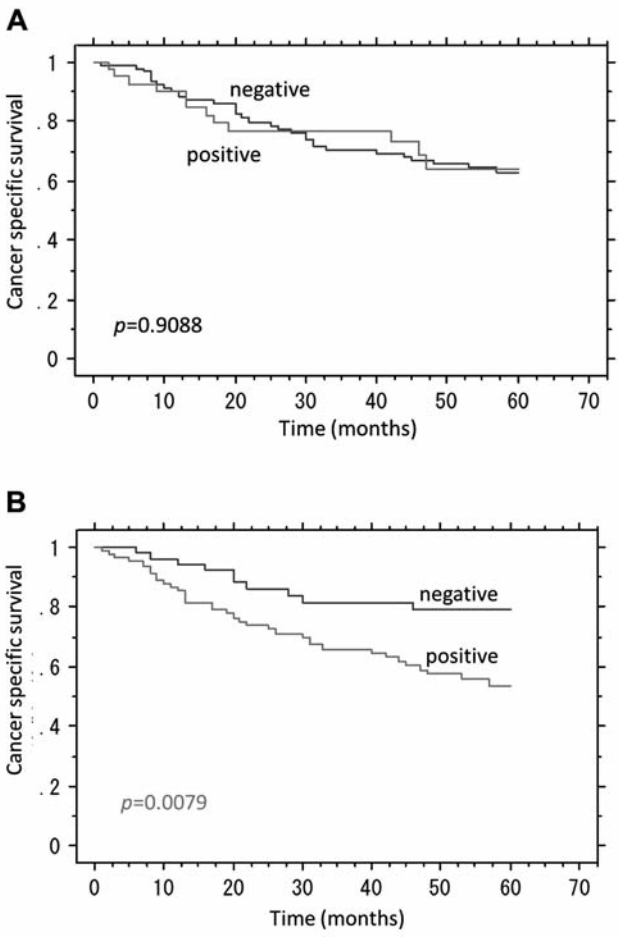

Figure 4. *Kaplan-Meier plots of cancer-specific survival based on (A) positive versus negative CXCL12 protein expression, and (B) positive versus negative CXCR4 protein expression in gastric tumor specimens.*

related to worse disease-free survival and overall survival. These findings suggest that gastric tumor cells with a high expression of CXCR4 and potentially activated by CXCL12, appear more likely to metastasize to the peritoneum and lymphatics, thereby hastening the progression of gastric cancer. We demonstrated that gastric cancer cells had two distinct immunostaining patterns for CXCR4: either cytomembrane or nuclear types, although both types were not associated with clinicopathological features or outcomes, similar to that reported for cases of hepatocellular carcinoma (25), breast cancer (26, 27) and nasopharyngeal carcinoma (28). CXCR4 is a transmembrane protein and it was originally thought that its expression would be detectable at the plasma membrane and cytoplasm; however, IHC analysis detected a nuclear pattern of CXCR4 expression.

Conversely, Spano *et al*. (29) showed that CXCR4-positive nuclear staining was associated with a significantly better outcome during early-stage non-small cell lung cancer (NSCLC). Yoshitake *et al*. (12) examined the localization of CXCR4 in colorectal cancer (CRC) cell lines and reported that CXCR4 immunoreactivity was detectable in both the cytomembrane and nuclear protein fraction, suggesting CXCR4 immunoreactivity in the nuclei of CRC cells was specific. Of note, CRC patients with nuclear-type CXCR4

expression showed more frequent lymph node metastasis, poor differentiation and worse outcomes than those expressing the cytomembrane type, suggesting the nuclear expression of CXCR4 might be associated with CRC progression. CXCR4 expression intensity was an important indicator that could be used to predict disease prognosis. Therefore, the CXCL12/CXCR4 axis has great potential as novel target molecules that could be used for gastric cancer therapy.

In summary, CXCL12 expression was not associated with survival. However, CXCR4 expression in gastric cancer was significantly associated with depth of invasion and poor survival. Thus, CXCR4 may be a potential biomarker for predicting poor survival of gastric cancer patients.

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Instructions to Authors 2014

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The principal aim of AR is to provide prompt publication (print and online) for original works of high quality, generally within 1-2 months from final acceptance. Manuscripts will be accepted on the understanding that they report original unpublished works on the cancer problem that are not under consideration for publication by another journal, and that they will not be published again in the same form. All authors should sign a submission letter confirming the approval of their article contents. All material submitted to AR will be subject to review, when appropriate, by two members of the Editorial Board and by one suitable outside referee. The Editors reserve the right to improve manuscripts on grammar and style.

The Editors and Publishers of AR accept no responsibility for the contents and opinions expressed by the contributors. Authors should warrantee due diligence in the creation and issuance of their work.

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Format. Two types of papers may be submitted: (i) Full papers containing completed original work, and (ii) review articles concerning fields of recognisable progress. Papers should contain all essential data in order to make the presentation clear. Reasonable economy should be exercised with respect to the number of tables and illustrations used. Papers should be written in clear, concise English. Spelling should follow that given in the “Shorter Oxford English Dictionary”.

Manuscripts. Submitted manuscripts should not exceed fourteen (14) pages (approximately 250 words per double - spaced typed page), including abstract, text, tables, figures, and references (corresponding to 4 printed pages). Papers exceeding four printed pages will be subject to excess page charges. All manuscripts should be divided into the following sections:
(a) *First page* including the title of the presented work [not exceeding fifteen (15) words], full names and full postal addresses of all Authors, name of the Author to whom proofs are to be sent, key words, an abbreviated running title, an indication “review”, “clinical”, “epidemiological”, or “experimental” study, and the date of submission. (Note: The order of the Authors is not necessarily indicative of their contribution to the work. Authors may note their individual contribution(s) in the appropriate section(s) of the presented work); (b) *Abstract* not exceeding 150 words, organized according to the following headings: Background/Aim - Materials and Methods/Patients and Methods - Results - Conclusion; (c) *Introduction;* (d) *Materials and Methods/Patients and Methods;* (e) *Results;* (f) *Discussion;* (g) *Acknowledgements;* (h) *References*. All pages must be numbered consecutively. Footnotes should be avoided. Review articles may follow a different style according to the subject matter and the Author's opinion. Review articles should not exceed 35 pages (approximately 250 words per double-spaced typed page) including all tables, figures, and references.

Figures. All figures (whether photographs or graphs) should be clear, high contrast, at the size they are to appear in the journal: 8.00 cm (3.15 in.) wide for a single column; 17.00 cm (6.70 in.) for a double column; maximum height: 20.00 cm (7.87 in.). Graphs must be submitted as photographs made from drawings and must not require any artwork, typesetting, or size modifications. Symbols, numbering and lettering should be clearly legible. The number and top of each figure must be indicated. Colour plates are charged.

Tables. Tables should be typed double-spaced on a separate page, numbered with Roman numerals and should include a short title.

References. Authors must assume responsibility for the accuracy of the references used. Citations for the reference sections of submitted works should follow the standard form of “Index Medicus” and must be numbered consecutively. In the text, references should be cited by number. Examples: 1 Sumner AT: The nature of chromosome bands and their significance for cancer research. Anticancer Res 1: 205-216, 1981. 2 McGuire WL and Chamnes GC: Studies on the oestrogen receptor in breast cancer. In: Receptors for Reproductive Hormones (O' Malley BW, Chamnes GC (eds.). New York, Plenum Publ Corp., pp 113-136, 1973.

Nomenclature and Abbreviations. Nomenclature should follow that given in "Chemical Abstracts", "Index Medicus", "Merck Index", "IUPAC –IUB", "Bergey's Manual of Determinative Bacteriology", The CBE Manual for Authors, Editors and Publishers (6th edition, 1994), and MIAME Standard for Microarray Data. Human gene symbols may be obtained from the HUGO Gene Nomenclature Committee (HGNC) (http://www.gene.ucl.ac.uk/). Approved mouse nomenclature may be obtained from http://www.informatics.jax.org/. Standard abbreviations are preferable. If a new abbreviation is used, it must be defined on first usage.

Clinical Trials. Authors of manuscripts describing clinical trials should provide the appropriate clinical trial number in the correct format in the text.
For International Standard Randomised Controlled Trials (ISRCTN) Registry (a not-for-profit organization whose registry is administered by Current Controlled Trials Ltd.) the unique number must be provided in this format: ISRCTNXXXXXXXX (where XXXXXXXX represents the unique number, always prefixed by "ISRCTN"). Please note that there is no space between the prefix "ISRCTN" and the number. Example: ISRCTN47956475.

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Ethical Policies and Standards. ANTICANCER RESEARCH agrees with and follows the "Uniform Requirements for Manuscripts Submitted to Biomedical Journals" established by the International Committee of Medical Journal Editors in 1978 and updated in October 2001 (www.icmje.org). Microarray data analysis should comply with the "Minimum Information About Microarray Experiments (MIAME) standard". Specific guidelines are provided at the "Microarray Gene Expression Data Society" (MGED) website. Presentation of genome sequences should follow the guidelines of the NHGRI Policy on Release of Human Genomic Sequence Data. Research involving human beings must adhere to the principles of the Declaration of Helsinki and Title 45, U.S. Code of Federal Regulations, Part 46, Protection of Human Subjects, effective December 13, 2001. Research involving animals must adhere to the Guiding Principles in the Care and Use of Animals approved by the Council of the American Physiological Society. The use of animals in biomedical research should be under the careful supervision of a person adequately trained in this field and the animals must be treated humanely at all times. Research involving the use of human foetuses, foetal tissue, embryos and embryonic cells should adhere to the U.S. Public Law 103-41, effective December 13, 2001.

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