The granulocyte/lymphocyte ratio as an independent predictor of tumour growth, metastasis and progression: Its clinical applications

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Abstract. Several investigators have suggested that the granulocyte/lymphocyte (G/L) ratio is a good indicator for the evaluation of the condition of a tumour-bearing host, although its prognotic significance is unclear. To further investigate the clinical applications of the G/L ratio, we injected $1x10^5$ and $1x10^6$ Lewis lung carcinoma cells (3LLc) into the feet of 4-week-old C57BL/6 mice separated into groups A, B, C and D ($1x10^5$ cells) and E, F, G and H ($1x10^6$ cells). For the observation of tumour metastasis and G/L ratio, the mice in groups A-D were sacrificed on days 11, 14, 17 and 21 after inoculation with the 3LLc cells, and the mice in groups E-H on days 7, 11, 14 and 17. The results suggest that in mice the number of granulocytes increases with time after 3LLc cell injection ($P<0.05$). We also retrospectively investigated the correlation between G/L ratio, clinicopathologic features and prognosis in 62 patients with gastric carcinoma. There was a significant correlation between the G/L ratio and tumour weight ($r=0.746$, $P<0.05$), as well as a significant difference between the G/L ratio and the extent of metastases ($P<0.05$). Additionally, the G/L ratio was significantly associated with lymph node metastasis and higher tumour stage, tumour progression ($P=0.017$) and 5-year survival ($P=0.013$). In conclusion, the G/L ratio is associated with tumour progression and shorter survival. The close correlation between G/L ratio and tumour stage or lymph node status suggests that it could be used to predict tumour metastasis, prognosis and overall survival in patients with gastric carcinoma before they undergo surgical treatment.

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Introduction

Several investigators have suggested that certain immune cells play an essential role in the progression of tumours, and are therefore good indicators of prognosis. Patients with lymphocyte infiltration around the tumour, for example, may have a good prognosis due to efficient natural killer (NK) cells, or because the lymphocytes result in lymphokine-activated tumour cell killing (1-3). In line with this thinking, leukocytosis is frequently observed in patients with tumours of an advanced stage, in postoperative recurrent cancers and in metastasis. In support of these findings, Ietomi (4) found that, unlike lymphocytes, granulocytes have many antigens in common with cancer cells, and that autologous NK cell activity is impaired with the emergence of an increasing number of granulocytes. Likewise, Ubukata *et al* (5) reported that granulocytes antagonize the anti-tumour activities of lymphocytes. In clinical settings, Tabuchi *et al* (6,7) treated tumour-bearing patients who had granulocytosis by selective granulocytapheresis (a procedure which can produce controlled depletion of granulocytes) and reported a resulting prolongation of survival. These observations indicate that the interaction between granulocytes and lymphocytes compromises tumour immunity in tumour-bearing hosts.

In light of the fact that elevated levels of granulocytes (with obvious activation behaviour) might impair the anti-tumour activity of lymphocytes, the granulocyte/lymphocyte (G/L) ratio, representing the relative number of each of these two major leukocyte populations, can indicate fluctuations in their numbers and their likely impact on the progression or prognosis of cancer. Hence the G/L ratio, which can be measured quite easily, should in the clinical setting be a valuable index (indicator of tumour progression) for determining which patients would benefit from surgery (4). Based on these concepts, the contribution of granulocytes and lymphocytes to the growth and metastasis of tumours, together with the G/L ratio, were the focus of this study. A mouse model for

tumour growth and metastases was used and a retrospective investigation of patients with gastric carcinoma was conducted to this end.

Materials and methods

Animals. The study was conducted in accordance with the standards established by the Guidelines for the Care and Use of Laboratory Animals of the Japan Immunoresearch Laboratories (JIMRO), with extra care taken to avoid animal suffering. Four-week-old C57BL/6N male mice, provided by JIMRO (Gunma, Japan) were used in all the experiments. Animals were bred in a standard laboratory setting and were allowed free access to food and water.

Cancer cell line. The 3LLc Lewis lung carcinoma cell line was kindly provided by the Cell Resource Center for Biomedical Research of the Institute of Development, Aging and Cancer, Tohoku University, Japan. Cells were maintained in a culture of Dulbecco's modified Eagle's medium supplemented with 10% foetal calf serum. Tumour cells were subcutaneously inoculated into the dorsum of mice feet, and tumours were freshly harvested when they had grown to ~1.5 cm in diameter (at ~2 weeks). Tumour cell suspensions were prepared in phosphate-buffered saline (PBS) by the passage of fresh tumour cell suspension through a sequential series of 18-, 22-, 27- and 30-gauge needles.

Patients. Patients (n=62) who had had a gastrectomy between 1999 and 2003 at the Fourth Department of Surgery, Tokyo Medical University Kasumigaura Hospital, were selected for the retrospective study. Those who had been lost to follow-up as well as those with synchronous or metachronous multiple cancers were excluded from the study. None of the patients had received preoperative chemotherapy or radiation therapy. The staging of gastric carcinoma was classified according to the 1997 tumour node metastasis (TNM) classification recommended by the International Union Against Cancer (5th edition). Patients comprised 44 men and 18 women with a mean age of 68.4±8.2 years. Overall survival was calculated from the date of diagnosis until the date of death or last follow-up. Patients who had died from causes unrelated to carcinoma with no evidence of the disease were exluded from the final data.

Experimental design

Experiment 1. Mice were randomly assigned to 8 groups (A-H). In groups A-D, the left foot dorsum of each mouse was subcutaneously injected with $1x10^5$ 3LL cells suspended in 0.1 ml PBS, while mice in groups E-H were injected with $1x10^6$ 3LL cells (10-fold higher). After implantation, all the mice were exsanguinated from the heart under general anaesthesia and sacrificed on days 11 (group A), 14 (group B), 17 (group C), 21 (group D), 7 (group E), 11 (group F), 14 (group G) and 17 (group H). Sequentially, tumours and lungs were removed and rinsed in 0.9% sodium chloride solution, then the weight of the implanted tumour and the number of peripheral blood leukocytes were measured. The number of lung metastatic colonies was determined by microscopic examination.

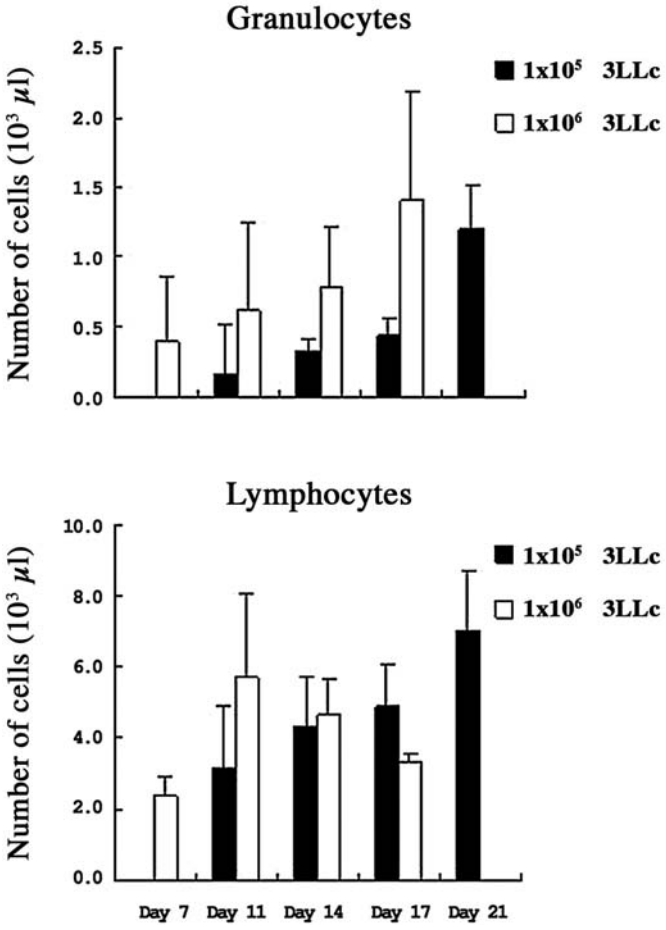

Figure 1. Fluctuation in the number of granulocytes and lymphocytes over time after implantation.

Experiment 2. Granulocyte and lymphocyte counts were collected at diagnosis and at 1 year, 6 months and 3 months before death. Patients were divided into two groups according to preoperative G/L ratio. A cutoff point of 4.0 for the G/L ratio was chosen as a representative upper reference level.

Statistical analysis. Testing of the statistical significance of the differences between groups was carried out with one-way ANOVA and multiple comparison tests, while the χ^2 test was used for the comparison of G/L ratio and clinicopathologic features. Survival curves were computed according to the Kaplan Meier method to determine differences between curves. P-values were calculated using the log-rank test. The prognostic significance of clinical and molecular parameters was examined by binary logistic regression. A P-value <0.05 was considered statistically significant.

Results

Effects of tumour implantation on mouse peripheral blood leukocytes. The number of granulocytes showed an upward trend over time after implantation in both the $1x10^6$ ($P<0.0001$) and $1x10^5$ ($P=0.035$) cell groups. In the $1x10^6$ cell groups, the number of granulocytes measured on days 11, 14 and 17 after implantation (groups F-H) was significantly increased in comparison with the control group (normal mice, 0.309±0.089),

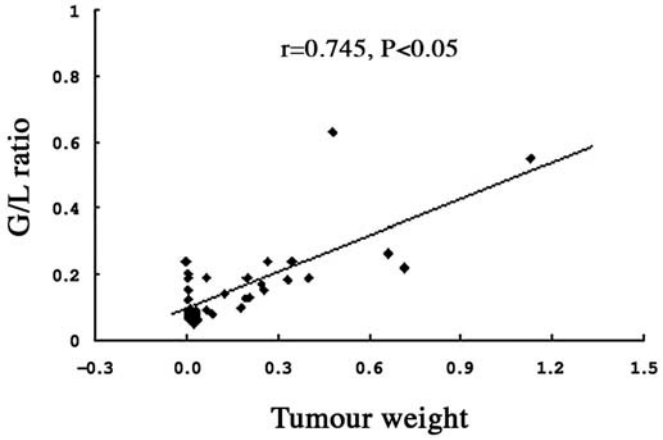

Figure 2. Positive linear correlation between tumour weight and the G/L ratio.

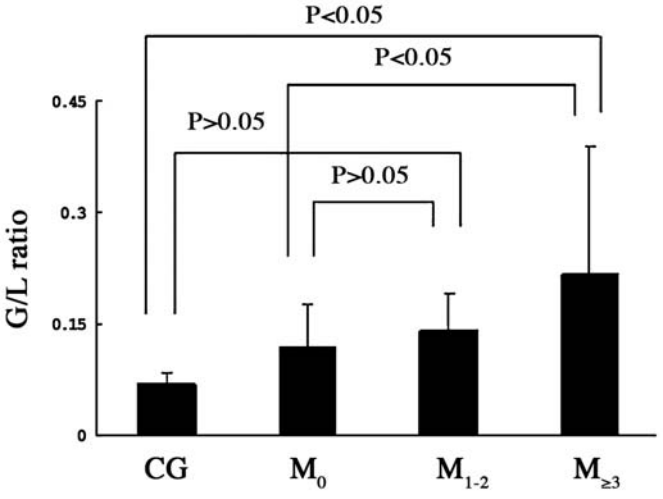

Figure 3. Increase in G/L ratio and extent of metastatic colonies in the lungs of mice. CG, control group; M_0, non-lung metastasis group; M_{1-2}, 1-2 lung metastasis-colony group; $M_{\geq 3}$, ≥3 lung metastasis-colony group.

while the number of lymphocytes peaked on day 11 after implantation, then decreased. In the $1x10^5$ cell groups, the number of granulocytes on days 11, 14 and 17 after implantation (groups A-C) showed no significant increase in comparison with the control group. However, on day 21 after implantation (group D), the number of granulocytes showed a significant increase in comparison with the control group. Furthermore, lymphocytes in the $1x10^5$ cell groups continued to increase until all mice had been sacrificed (Fig. 1).

Relationship between G/L ratio and tumour progression in mice. The relationship between tumour weight and the G/L ratio was investigated in all the mice, and a positive linear correlation (r=0.746, P<0.05) was found (Fig. 2). Based on the results of lung metastasis, data were divided into the following groups: CG, control; M_0, non-lung metastasis; M_{1-2}, 1-2 lung metastasis colonies; $M_{\geq 3}$, ≥3 lung metastasis colonies. The G/L ratio showed a positive correlation with the number of lung metastases (P=0.013) using one-way ANOVA and, using multiple comparison tests, a significant difference between the CG or M_0 groups and the $M_{\geq 3}$ group (P=0.002 and 0.018, respectively). However, no significant difference was found between the M_{1-2} and the GC or M_0 groups (P>0.05, Fig. 3).

Table I. The G/L ratio according to tumour features.

Variable	G/L ratio <4.0	G/L ratio ≥4.0	P-value
Age			NS
<60	8	1	
≥60	44	9	
Gender			NS
Male	38	6	
Female	14	4	
Depth of invasion			NS
Mucosa/submucosa	18	1	
Muscle layer	14	2	
Subserosa/serosa exposed	20	7	
Histology			NS
Well/moderate	36	5	
Poor/mucinous	16	5	
Lymph node metastasis			0.03
N0	22	3	
N1	16	1	
N2	12	3	
N3	2	3	
Stage			<0.0001
I	24	1	
II	6	3	
III	19	1	
III	3	5	

NS, not significant; G/L ratio, granulocyte/lymphocyte ratio.

Relationship between G/L ratio and clinicopathologic features, tumour progression and survival. The 4.0 cutoff point for the G/L ratio was set as the upper level. According to the preoperative G/L ratio, patients were divided into a G/L <4.0 group (n=52) and a G/L ≥4.0 group (n=10). Analysis revealed a G/L of ≥4.0 to be significantly associated with lymph node metastasis and higher tumour stage (Table I). At the end of the follow-up period, the 5-year survival rate was 43.5% (27/62) for all patients. Kaplan Meier analysis suggested that the survival rate of patients with a G/L ≥4.0 was significantly lower than that of patients with a G/L <4.0 (P=0.013, Fig. 4). In multivariate analysis, tumour stage, and not the G/L ratio, was statistically significant for survival (Table II). Next, in 20 patients who had died of gastric carcinoma with a survival time of >1 year, the peripheral granulocyte and lymphocyte counts were determined at 1 year, 6 months and 3 months before death. One-way ANOVA suggested that the G/L ratio had a significant positive correlation with tumour progression (P=0.017, Fig. 5).

Table II. Logistic regression analysis for survival.

Variable	Survival (%)	Univariate[a]	Multivariate[b]	
		P-value	RR (95% CI)	P-value
Age		NS		NS
<60	66.7			
≥60	53.8			
Gender		NS		NS
Male	59.1			
Female	47.1			
Depth of invasion		<0.0001		NS
Mucosa/submucosa	94.7			
Muscle layer	75.0			
Subserosa/serosa exposed	15.4			
Lymph node metastasis		<0.0001		NS
Present	91.7			
Absent	32.4			
Histology		0.0230		NS
Well/moderate	65.9			
Poor/mucinous	35.0			
Macroscopic type		<0.0001		NS
Early carcinoma	94.4			
Advanced carcinoma	39.5			
G/L ratio		0.0120		NS
<4.0	62.7			
≥4.0	20.0			
Stage		<0.0001	0.117 (0.045-0.301)	<0.0001
I	96.0			
II	62.5			
III	25.0			
IV	12.5			

NS, not significant; RR, relative risk; CI, confidence interval; G/L ratio, granulocyte/lymphocyte ratio. [a]Log-rank test; [b]logistic regression model analysis.

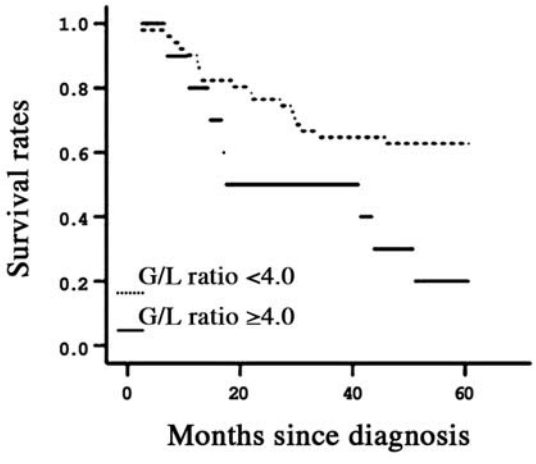

Figure 4. Survival curves of patients with gastric carcinoma. The 5-year survival rate of patients with a G/L ratio ≥4.0 was significantly lower than that of patients with a G/L ratio <4.0 (P=0.013).

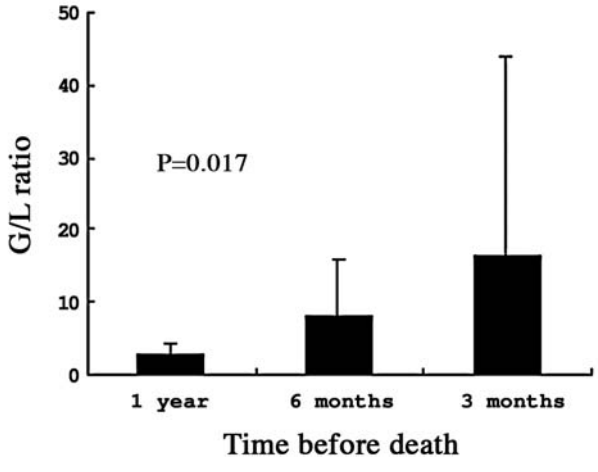

Figure 5. G/L ratio and tumour progression.

Discussion

We set out to clarify the role of peripheral granulocytes and lymphocytes in tumour progression. Several investigators have suggested that the G/L ratio is a good indicator for the evaluation of the condition of a tumour-bearing host (4,6), although the correlation between granulocytes and lymphocytes or between the G/L ratio and clinicopathologic factors is unclear (8).

In the $1x10^6$ cell mice in our animal model, the number of lymphocytes tended, after a transient increase, to decrease from day 11 after implantation, while tumour weight and the number of granulocytes exhibited an obvious unremitting increase. The number of granulocytes measured at each stage after implantation (groups E-H) was significantly increased in comparison with the control group (normal mice, 0.309± 0.089). In contrast, in the $1x10^5$ cell groups, a decrease in lymphocytes was not observed. Rather, lymphocytes increased until all the mice had been sacrificed. Furthermore, although the number of granulocytes had an uptrend with time after implantation, on days 11, 14 and 17 after implantation they were not significantly increased as compared to the control group. However, on day 21 after implantation, the number of granulocytes showed a significant increase in comparison with the control group and with groups A-C. From these findings, it can be extrapolated that the number of lymphocytes in the $1x10^5$ cell groups decreased after day 21 because they were suppressed by the significant increase in the number of granulocytes. Our results are supported by previous studies involving granulocyte and lymphocyte co-incubation with tumour or effector cells *in vitro,* which indicate that granulocytes suppress the anti-tumour activities of lymphocytes and lymphokine-activated tumour cell killing (5,9,10).

Based on the above, we believe that elevated levels of granulocytes promote tumour growth by antagonizing tumour-suppressing lymphocytes, and that the fluctuation in granulocyte number is not always in line with that of lymphocytes. Additionally, a positive linear correlation was found to exist between tumour weight and the G/L ratio. We consequently believe that the higher the G/L ratio, the weaker the anti-tumour efficiency of the host immune mechanism will be.

To assess the extent of tumour metastasis in relation to the G/L ratio, we analyzed the correlation between the two. Our results revealed that the G/L ratio is clearly associated with tumour metastasis, especially in the $M_{\geq 3}$ group.

Tumour cells are known to produce various cytokines and chemokines that can attract and promote leukocyte transformation into diverse leukocyte populations. Each of these can then produce an array of cytokines of its own to escape the immune control mechanism of the host (11,12). As a result, leukocytosis is frequently observed in patients with advanced stage tumours or postoperative recurrence and metastasis (13).

Riesco (14) has suggested that a high neutrophil count is correlated with poor prognosis in patients with a variety of cancers, including that of the breast, head and neck, and sarcoma. More recent studies in a variety of advanced cancers have shown a high count of peripheral neutrophils to be an independent prognostic factor for short survival (15-18). However, few studies have been conducted on the prognostic role of the G/L ratio.

In humans, granulocytes show an increase in the daytime (daytime rhythm), while T-cells, B-cells, alphabet T-cells and $CD4^+$ lymphocytes show an increase at night (19). Fluctuations in granulocyte number are not always in line with those of lymphocytes. The G/L ratio as a relative value can correctly reflect fluctuations between granulocytes and lymphocytes, and can reflect the anti-tumour efficiency of the host immune mechanism more exactly than neutrophils can. We therefore propose that the G/L ratio could be used as a predictor in the clinical setting.

We next investigated the G/L ratio in relation to clinicopathologic factors. Upon setting a cutoff point of 4.0 for the G/L ratio as a representative upper reference level, the results indicated that a G/L ≥4.0 is significantly associated with lymph node metastasis and higher tumour stage. Meanwhile, the G/L ratio measured at each stage (before death) showed a significant difference, suggesting that it is significantly associated with tumour growth, progression and metastasis. Regarding the 5-year survival rate of the patients, we found that a G/L ≥4.0 was significantly associated with shorter survival. However, multivariate analysis revealed that only higher tumour stage was significantly associated with poor prognosis. Because of the close correlation between G/L ratio and tumour stage, multivariate analysis in which lymph node status and tumour stage were excluded was also performed, and the G/L ratio was found to be an independent predictor of survival ($P<0.05$).

In conclusion, our experimental tumour mouse model demonstrated that elevated peripheral granulocytes are associated with the growth and metastasis of tumours, perhaps because they compromise the natural anti-tumour function of lymphocytes. However, although the G/L ratio has been associated with tumour progression and shorter survival in humans, multivariate analysis in the current study did not reveal it to be associated with poor prognosis. The close correlation between G/L ratio and tumour stage or lymph node status leads us to believe that the G/L ratio could help predict tumour metastasis, prognosis and overall survival in patients with gastric carcinoma, before they undergo surgical treatment.

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References

1. Aaltomaa S, Lipponen P, Eskelinen M, *et al*: Lymphocyte infiltrates as a prognostic variable in female breast cancer. Eur J Cancer 28: 859-864, 1992.
2. Kawata A, Une Y, Hosokawa M, *et al*: Tumor-infiltrating lymphocytes and prognosis of hepatocellular carcinoma. Jpn J Clin Oncol 22: 256-263, 1992.
3. Slootweg PJ, De Pagter M, De Weger RA, *et al*: Lymphocytes at tumor margins in patients with head and neck cancer. Relationship with tumor size, human lymphocyte antigen molecules, and metastasis. Int J Oral Maxillofac Surg 23: 286-289, 1994.
4. Ietomi K: A study on the role of granulocytes in carcinoma-bearing hosts - G/L ratio as a new host indicator. Nippon Gan Chiryo Gakkai Shi 25: 662-671, 1990.
5. Ubukata H, Tabuchi T, Soma T, *et al*: The role of granulocyte in the immune response network against cancer. Biotherapy 6: 776-778, 1992.
6. Tabuchi T, Ubukata H, Sato S, *et al*: Granulocytapheresis as a possible cancer treatment. Anticancer Res 15: 985-990, 1995.

7. Tabuchi T, Ubukata H, Saniabadi AR, *et al*: Granulocyte apheresis as a possible new approach in cancer therapy: A pilot study involving two cases. Cancer Detect Prev 23: 417-421, 1999.
8. Toge T and Yamaguchi Y: Protein-bound polysaccharide increases survival in resected gastric cancer cases stratified with a preoperative granulocyte and lymphocyte count. Oncol Rep 7: 1157-1161, 2000.
9. Petrie HT, Klassen LW, Kay HD, *et al*: Inhibition of human cytotoxic T lymphocyte activity in vitro by autologous peripheral blood granulocytes. J Immunol 134: 230-234, 1985.
10. Shau HY and Kim A: Suppression of lymphokine-activated killer induction by neutrophils. J Immunol 141: 4395-4402, 1988.
11. Kuper H, Adami HO, Trichopoulos D, *et al*: Infections as a major preventable cause of human cancer. J Intern Med 248: 171-183, 2000.
12. Wahl LM and Kleinman HK: Tumor-associated macrophages as targets for cancer therapy. J Natl Cancer Inst 90: 1583-1584, 1998.
13. Shimasaki AK, Hirata K, Kawamura T, *et al*: The level of serum granulocyte colony-stimulating factor in cancer patients with leukocytosis. Intern Med 31: 861-865, 1992.
14. Riesco A: Five-year cancer cure: relation to total amount of peripheral lymphocytes and neutrophils. Cancer 25: 135-140, 1970.
15. Atzpodien J, Royston P, Wandert T, *et al*: Metastatic renal carcinoma comprehensive prognostic system. Br J Cancer 88: 348-353, 2003.
16. Négrier S, Escudier B, Gomez F, *et al*: Prognostic factors of survival and rapid progression in 782 patients with metastatic renal carcinomas treated by cytokines: a report from the Groupe Français d'Immunothérapie. Ann Oncol 13: 1460-1468, 2002.
17. Donskov F and von der Maase H: Impact of immune parameters on long-term survival in metastatic renal cell carcinoma. J Clin Oncol 24: 1997-2005, 2006.
18. Schmidt H, Suciu S, Punt CJ, *et al*: Pretreatment levels of peripheral neutrophils and leukocytes as independent predictors of overall survival in patients with American Joint Committee on Cancer Stage IV Melanoma: results of the EORTC 18951 Biochemotherapy Trial. J Clin Oncol 25: 1562-1569, 2007.
19. Suzuki S, Toyabe S, Moroda T, *et al*: Circadian rhythm of leucocytes and lymphocytes subsets and its possible correlation with the function of the autonomic nervous system. Clin Exp Immunol 10: 500-508, 1997.