Urological Oncology

The Relationship between B7-H4 Expression and Clinicopathological Characteristics in Clinical Stage T1 Conventional Renal Cell Carcinoma

Suk Gun Jung, Kyung Un Choi[1], Sang Don Lee, Zeong Zoo Lee, Moon Kee Chung

Departments of Urology and [1]Pathology, Pusan National University Yangsan Hospital, Pusan National University School of Medicine, Yangsan, Korea

Purpose: The diagnosis of clinically early-stage (T1) renal cell carcinoma (RCC) has increased. The present study evaluated the association of B7-H4 expression on the pathological outcome and recurrence of carcinoma in the T1 stage of RCC.

Materials and Methods: Among patients who underwent partial or radical nephrectomy after diagnosis of T1 stage RCC during the period of January 2000 to March 2007, 102 pathologically confirmed cases of clear cell carcinoma were included in this study. The patients' medical records were reviewed retrospectively. For the immunohistochemical staining tests, the B7-H4 antibody (Abbiotec 1:500) was used, and clinicopathological characteristics were analyzed.

Results: The mean age of the patients (39 males: 38.2%, 63 females: 61.8%) was 53.0± 12.0 years (range, 31-74 years), and the mean follow-up time was 33.4±21.0 months (range, 6-84 months). B7-H4 expression was positive in 18 cases and negative in 84 cases. Recurrence during the follow-up period occurred in 5 cases in the group with positive B7-H4 expression and in 7 cases in the group with negative B7-H4 expression, respectively (p=0.035). In the univariate analysis, a statistically significant relationship was observed only for the presence of B7-H4 expression (p=0.0019). In the multivariate analysis, other than the expression of B7-H4, cancer size and TNM stage had effects on the recurrence of cancer.

Conclusions: For clear cell RCC, B7-H4 expression had a critical impact on the prognosis of the patients, particularly on the recurrence of the carcinoma in patients with clinical stage T1 RCC.

Key Words: Prognosis; Recurrence; Renal cell carcinoma

INTRODUCTION

The classification of patients with renal cell carcinoma (RCC) by the level of risk for local recurrence, prognosis, and survival is important for patients and clinicians to consider before surgery so that appropriate treatment and follow-up can be provided. Several anatomical, clinical, histological, and molecular factors have been reported as useful for the prediction of patient prognosis [1]. Since 2001, numerous studies have been performed on a variety of clinical and pathological factors that can be used to determine a more accurate prognosis [2,3]. More recently, the assessment of molecular genetic characteristics has become important for the prediction of tumor-specific survival. Many genes and the associated proteins have been studied for their association with patient prognosis [4]. For example, a recent study from the Mayo Clinic reported that the expression of the B7-H4 protein was detected in a diverse group of malignant tumors including RCC; this protein has been associated with blocking of antitumor mechanisms [5]. B7-H4 was first reported by Chen et al in 2003; it is the ligand of the B7 protein and a suppressor of T cell mediated

immunity [6-8]. The B7-H4 protein is overexpressed in lung tumors, breast tumors, and ovarian tumors [9].

RCC has been classified as an immunogenic tumor, with lymphocyte infiltration. On occasion, complete disappearance of metastatic tumors, after the surgical removal of primary tumors, has been observed. However, only 5% to 10% of cases of advanced RCC respond to cytokine-based immunotherapy [10]. The mechanism associated with the ability of renal cancer cells to evade antitumor immunity is unknown and the focus of many current studies.

Currently, due to the increased use of radiological diagnostic techniques and the interest in prevention, the clinical diagnosis of early-stage RCC has increased. The goal of this study was to examine the association of the expression of B7-H4 with the pathological characteristics of RCC, patient prognosis, and disease recurrence in patients with stage T1 RCC.

MATERIALS AND METHODS

1. Materials
Among patients diagnosed with clinical stage T1 RCC who underwent a partial or radical nephrectomy from January 2000 to March 2007, 102 patients were confirmed to have clear cell tumors by the postsurgical pathological evaluation.

2. Clinical and pathological characteristic
Clinical and pathological characteristics were analyzed retrospectively on the basis of information in the patients' medical records. The data collected included age at the time of surgery, gender, tumor size at the time of diagnosis, and the presence or absence of symptoms. Patients with an abdominal lump, abdominal pain, macroscopic hematuria, a varicocele, or weight loss were considered symptomatic at the time of diagnosis. The histological type of tumor, TNM disease stage, nuclear grade, presence or absence of tumor necrosis, and presence or absence of lymphovascular invasion were evaluated.

The definitions of disease progression included local recurrence, lymph-node metastasis, and distant metastasis. However, in this study, no cases of local recurrence were identified.

3. Immunohistochemical staining
Sections 4 μm in thickness were obtained from paraffin-embedded tissues, attached to coated slides, incubated in a 60°C oven for 1 hour, deparaffinized by repeated treatment with xylene for 5 minutes four times, dehydrated by several steps of alcohol, and washed with distilled water. For antigenic recovery, the slides were soaked in 0.01 M citrate buffer and boiled in a microwave for approximately 10 minutes. Afterwards, the slides were cooled at room temperature for 30 minutes, washed with washing buffer for 3 minutes three times, and reacted in 3% H_2O_2 solution for 5 minutes to remove intrinsic peroxidase. The slides were washed with wash buffer four times, reacted with the first B7-H4 antibody (Abbiotec 1:500, rabbit polyclonal antibody) overnight at 4°C, washed four times, and reacted with horseradish peroxidase polymer conjugate for 10 minutes. Subsequently, the slides were washed with wash buffer four times, stained with diaminobenzidine, counterstained with hematoxylin, sealed, and examined under the light microscope.

The sporadic expression of B7-H4 protein in the distal convoluted tubules was considered the positive control group. Cases in which expression of the B7-H4 protein was detected in more than 10% of the tumor cells were considered positive, and cases in which expression was less than 10% were considered negative [11]. The results of the staining were interpreted by one pathologist. The tumor cells that were stained reddish brown with B7-H4 antibody were read as positive (Fig. 1).

4. Statistical analysis
The results were divided into groups with and without expression of B7-H4 and clinical and pathological characteristics were analyzed. For the analysis of recurrence-free
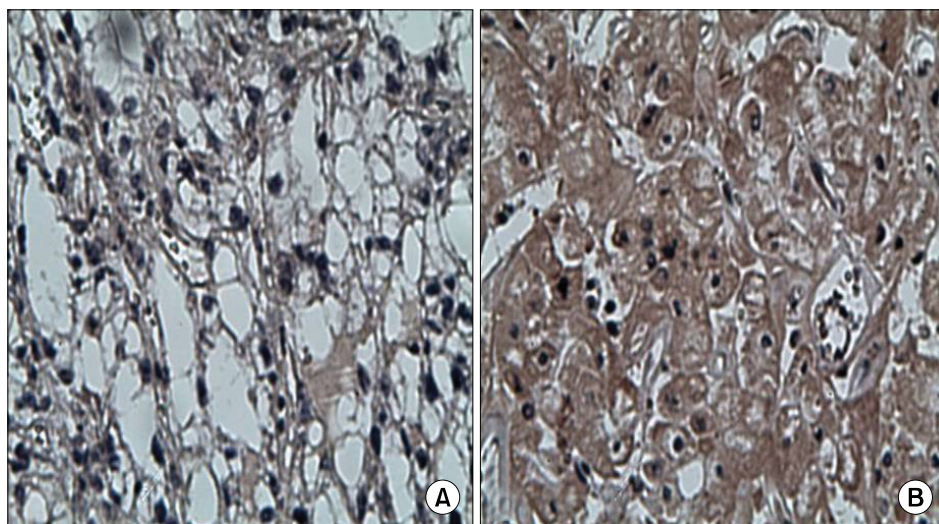

FIG. 1. Immunohistochemical staining of B7-H4. (A) Negative, (B) Positive (H&E, x200).

survival, univariate analysis, Kaplan-Meier, and the log-rank tests were performed. For the multivariate survival analysis, Cox's proportional hazard regression model was performed. The factors considered in the analysis included recurrence, age, gender, symptoms at the time of diagnosis, tumor size at the time of diagnosis, pathological disease stage, nuclear grade, lymphovascular invasion, and the expression of B7-H4. SPSS ver. 14.0 (SPSS Inc., Chicago, IL, USA) for Windows was used for the statistical analysis, and p-values less than 0.05 were considered statistically significant.

RESULTS

1. Relationship between the expression of B7-H4 and clinical and pathological characteristics

The mean age of the study group was 53.0±12.0 years (range, 31-74 years). The mean postsurgical follow-up period was an average of 33.4±21.0 months (range, 6-84 months). Males and females accounted for 39 patients (38.2%) and 63 patients (61.8%), respectively. The immunohistochemical staining showed that the group with expression of B7-H4 and the group without expression included 18 (17.6%) and 84 patients (82.4%), respectively. In the group with expression of B7-H4, 8 patients had a partial nephrectomy, and in the group without B7-H4 expression, 57 patients had a partial nephrectomy. Only 1 patient who had a partial nephrectomy had positive surgical margins; however, this patient did not have disease recurrence.

There was no significant difference between the groups with and without B7-H4 expression with regard to age, gender, symptoms at the time of diagnosis, tumor size at the time of diagnosis, TNM disease stage, lymphovascular invasion, or nuclear grade (Table 1).

2. Relationship between the expression of B7-H4 and recurrence rate

Among the 102 patients, in 5 out of 18 patients (27.8%) who were positive for the expression of B7-H4, lymph node metastasis and distant metastasis developed during the follow-up period. Among 84 patients negative for the expression of B7-H4, 7 patients (8.3%) developed disease recurrence; there was a higher recurrence rate in the B7-H4-positive group (p=0.035). Among the factors studied for their association with disease recurrence, only the expression of B7-H4 showed a statistically significant relationship by univariate analysis (Fig. 2). The multivariate survival analysis for postsurgical disease recurrence showed that expression of B7-H4, tumor size, and disease stage were associated with disease recurrence (Table 2).

DISCUSSION

Several types of genes, proteins, and growth factors have been associated with the prognosis of patients who have RCC [4]. One such factor, carbonic anhydrase IX (CAIX), is a prognostic factor that has been widely investigated. By

TABLE 1. Clinical and pathologic features by tumor B7-H4 expression

| Variables	n	B7-H4 antibody staining, n (%)		p-value[a]
		Positive	Negative	
Age (yr)				0.897
<65	76	10 (13.2)	66 (86.8)	
≥65	26	8 (30.8)	18 (69.2)	
Sex				0.577
Male	39	13 (33.3)	26 (66.7)	
Female	63	5 (7.9)	58 (92.1)	
Symptoms at presentation				0.461
Absent	84	17 (20.2)	67 (79.8)	
Present	18	1 (5.6)	17 (94.4)	
Primary tumor size (cm)				0.239
≤4	67	7 (10.4)	60 (89.6)	
4-7	35	11 (31.4)	24 (68.6)	
Primary tumor classification				0.19
pT1a	64	7 (10.9)	57 (89.1)	
pT1b	32	8 (25)	24 (75)	
pT3a	6	3 (50)	3 (50)	
Nuclear grade				0.08
1-2	82	12 (14.6)	70 (85.4)	
3-4	20	6 (30)	14 (70)	
Lymphovascular invasion				0.949
Absent	97	17 (17.5)	80 (82.5)	
Present	5	1 (20)	4 (80)	

[a]: Pearson's chi-square test
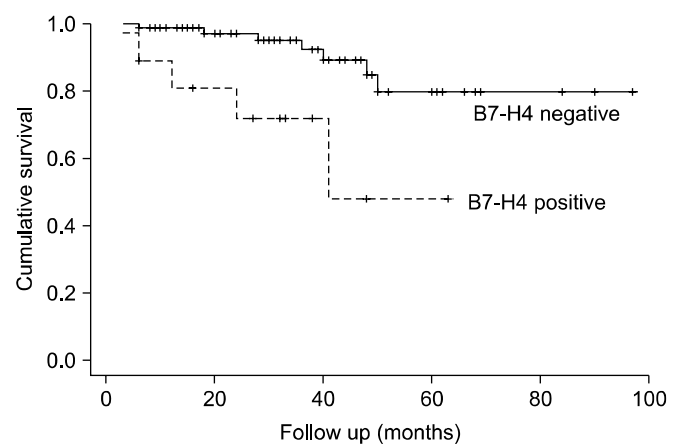
FIG. 2. Recurrence-free survival of the patients with renal cell carcinoma according to B7-H4 expression status (p=0.0019).

controlling intra- as well as extracellular acidity, CAIX plays an important role in the adaptation of cells to hypoxic environments. According to Bui et al, CAIX was shown to be an independent prognostic factor for the tumor-specific survival rate, and regardless of the disease stage of the tumors or their histological grade, the probability of death in

TABLE 2. Relationship between various parameters and recurrence-free survival

Variables	Univariate analysis	Multivariate analysis	
	p-value[a]	Hazard ratio	p-value[a]
Age	0.289	2.607	0.106
Sex	0.457	0.491	0.484
Symptoms at presentation	0.955	0.440	0.507
Primary tumor size	0.574	5.404	0.020
Stage	0.279	3.909	0.048
Nuclear grade	0.135	0.130	0.719
Lymphovascular invasion	0.678	0.414	0.520
B7-H4 staining	0.002	7.354	0.007

[a]: univariate analysis was done by using the Kaplan-Meier curve and the log-rank test and multivariate analysis was done by using the Cox's proportional hazard regression model

cases with a low expression of CAIX was approximately three times that than in cases with a high expression [12]. However, in another study performed on 730 patients with clear cell RCC, the multivariate analysis did not confirm that CAIX was an independent prognostic factor [13].

Aberrant expression of the insulin-like growth factor mRNA binding protein (IMP) family member IMP-3 has been demonstrated in several human malignancies [14]. The role of IMP-3 in tumorigenesis is thought to involve the promotion of cellular proliferation. IMP-3 expression was a significant independent predictor of metastasis-free and overall survival after multivariate adjustment. These findings were validated by Hoffmann et al in a study of 716 consecutive patients with clear cell RCC examined for IMP-3 expression; 88% (629 of 716) of the specimens examined had pathologically localized disease at the time of nephrectomy [15]. Among the pathologically localized tumors, 26% (163 of 629) stained positive for IMP-3. The 5- and 10-year progression-free survival rates were significantly lower in patients with IMP-3-positive tumors than in patients with IMP-3-negative tumors (51% and 41% vs. 89% and 82%, respectively).

Survivin is an antiapoptotic protein that is overexpressed in almost all human malignancies; it is rarely detected in normal tissue. Survivin is believed to inhibit both the intrinsic and extrinsic caspase cascades. Survivin expression has been correlated with several adverse pathological features of clear cell RCC, including tumor size, nuclear grade, TNM classification, presence of metastatic disease, coagulative tumor necrosis, and sarcomatoid differentiation [16]. Moreover, in a series reported by Parker et al, 312 patients with high survivin expression were twice as likely to die from clear cell RCC as were patients with low survivin expression [16].

The B7 family plays an important role in the regulation of antigen-specific T cell-mediated immune reactions. The expression of such proteins is limited to phagocytic and lymphoid cells [17]. The expression of B7-H4 and B7-H1 in several tumors, including clear cell RCC, has been reported; they have been associated with blocking effective anti-tumor immune mechanisms and with an increased risk of tumor-specific death [18]. Several studies have demonstrated the expression of B7-H4 in clear cell tumors of the kidneys [19]; these findings were confirmed in this study. The results of this study showed that tumor recurrence was more common in patients with B7-H4 expression.

B7-H4 has been shown to be a suppressor of the T cell-mediated immune response [6-8]. Several studies have reported that B7-H4 acts directly on apoptotic mechanisms associated with tumor cells. In particular, in ovarian tumors, B7-H4 has been reported to accelerate tumor formation. In breast tumors, the expression of B7-H4 mRNA and protein were blocked *in vitro*, and this was associated with the acceleration of apoptosis of tumor cells [20]. Therefore, B7-H4 appears to act by two different mechanisms. One is as a negative regulator of cell-mediated antitumor activity, and the other is by the unresponsiveness of tumor cells to apoptosis. B7-H4 suppresses the proliferation of CD4 and CD8 T cells as well as the secretion of cellular hormones. The receptors are thought to be the B and T lymphocyte attenuator (BTLA) expressed on activated T cells [21]. These mechanisms enhance disease progression and tumor activity. However, studies on the mechanism of B7-H4 activity are in early stages, and further research is needed.

The results of this study confirmed the expression of B7-H4 in clear cell RCC. Previous studies reported the sporadic expression of B7-H4 in the distal convoluted tubules [22]. However, the expression of B7-H4 in the proximal convoluted tubules has not been reported [23,24]. In addition, the expression of B7-H4 has been associated with lymphocyte infiltration and a poor prognosis in patients with cancer [25]. Moreover, in this study, the expression of B7-H4 was shown to increase the risk of recurrence in patients with localized RCC.

Currently, the role of genetic factors and molecular markers has been limited in the diagnosis and management of patients with renal cell cancer. However, in the future, advancements in imaging technology as well as immunotherapy in combination with genetic studies might improve the treatment and management of patients with renal cell cancer, such as with sunitinib and sorafenib [26].

Molecular biomarkers can assist with the diagnosis of a malignancy, may represent potential therapeutic targets, and may offer a means to monitor disease recurrence after treatment. Moreover, selected biomarkers may prove useful when incorporated into existing risk stratification models to help clinicians more accurately counsel patients regarding disease prognosis. Clinically, the use of molecular markers has several problems. The molecular markers require immunohistochemical staining of tumor tissues. Biological markers that could be used on serum and urine samples would be more accessible for the assessment of response to therapy as well as for tumor surveillance [27].

The limitations of this study include the following. This study was a retrospective chart review. According to previous reports [5], the expression of B7-H4 was 59.0%, and in this study it was 17.6%. This low expression rate is thought to be due to the immunohistochemical staining method used to assess the expression of B7-H4 in paraffin-embedded tissues. Many previous studies used fresh frozen samples. In addition, the study subjects in this report represented clinical disease limited to stage T1. Furthermore, the sample size was relatively small.

CONCLUSIONS

The results of this study showed that the expression of B7-H4 was associated with the prognosis of patients with clear cell RCC and recurrence in patients with stage T1 tumors. Although multiple current prognostic models based on clinical and pathological variables exist, further improvements in the accuracy of such models may be achieved with the development and implementation of molecular biomarkers. The expression of B7-H4 might be used to manage patients with RCC and as a target for novel treatments.

Conflicts of Interest
The authors have nothing to disclose.

REFERENCES

1. Lam JS, Pantuck AJ, Belldegrun AS, Figlin RA. Protein expression profiles in renal cell carcinoma: staging, prognosis, and patient selection for clinical trials. Clin Cancer Res 2007;13: 703s-8.
2. Kattan MW, Reuter V, Motzer RJ, Katz J, Russo P. A postoperative prognostic nomogram for renal cell carcinoma. J Urol 2001;166:63-7.
3. Frank I, Blute ML, Cheville JC, Lohse CM, Weaver AL, Zincke H. An outcome prediction model for patients with clear cell renal cell carcinoma treated with radical nephrectomy based on tumor stage, size, grade and necrosis: the SSIGN score. J Urol 2002;168: 2395-400.
4. Lam JS, Leppert JT, Figlin RA, Belldegrun AS. Role of molecular markers in the diagnosis and therapy of renal cell carcinoma. Urology 2005;66(5 Suppl):1-9.
5. Krambeck AE, Thompson RH, Dong H, Lohse CM, Park ES, Kuntz SM, et al. B7-H4 expression in renal cell carcinoma and tumor vasculature: associations with cancer progression and survival. Proc Natl Acad Sci U S A 2006;103:10391-6.
6. Zang X, Loke P, Kim J, Murphy K, Waitz R, Allison JP. B7x: a widely expressed B7 family member that inhibits T cell activation. Proc Natl Acad Sci U S A 2003;100:10388-92.
7. Prasad DV, Richards S, Mai XM, Dong C. B7S1, a novel B7 family member that negatively regulates T cell activation. Immunity 2003;18:863-73.
8. Sica GL, Choi IH, Zhu G, Tamada K, Wang SD, Tamura H, et al. B7-H4, a molecule of the B7 family, negatively regulates T cell immunity. Immunity 2003;18:849-61.
9. Choi IH, Zhu G, Sica GL, Strome SE, Cheville JC, Lau JS, et al. Genomic organization and expression analysis of B7-H4, an immune inhibitory molecule of the B7 family. J Immunol 2003;171: 4650-4.
10. Fyfe G, Fisher RI, Rosenberg SA, Sznol M, Parkinson DR, Louie AC. Results of treatment of 255 patients with metastatic renal cell carcinoma who received high-dose recombinant interleukin-2 therapy. J Clin Oncol 1995;13:688-96.
11. Thompson RH, Gillett MD, Cheville JC, Lohse DM, Dong H, Webster WS, et al. Costimulatory B7-H1 in renal cell carcinoma patients: indicator of tumor aggressiveness and potential therapeutic target. Proc Natl Acad Sci U S A 2004;101:17174-9.
12. Bui MH, Seligson D, Han KR, Pantuck AJ, Dorey FJ, Huang Y, et al. Carbonic anhydrase IX is an independent predictor of survival in advanced renal clear cell carcinoma: implications for prognosis and therapy. Clin Cancer Res 2003;9:802-11.
13. Leibovich BC, Sheinin Y, Lohse CM, Thompson RH, Cheville JC, Zavada J, et al. Carbonic anhydrase IX is not an independent predictor of outcome for patients with clear cell renal cell carcinoma. J Clin Oncol 2007;25:4757-64.
14. Zheng W, Yi X, Fadare O, Liang SX, Martel M, Schwartz PE, et al. The oncofetal protein IMP3: a novel biomarker for endometrial serous carcinoma. Am J Surg Pathol 2008;32:304-15.
15. Hoffmann NE, Sheinin Y, Lohse CM, Parker AS, Leibovich BC, Jiang Z, et al. External validation of IMP3 expression as an independent prognostic marker for metastatic progression and death for patients with clear cell renal cell carcinoma. Cancer 2008;112:1471-9.
16. Parker AS, Kosari F, Lohse CM, Houston Thompson R, Kwon ED, Murphy L, et al. High expression levels of survivin protein independently predict a poor outcome for patients who undergo surgery for clear cell renal cell carcinoma. Cancer 2006;107:37-45.
17. Dong H, Zhu G, Tamada K, Chen L. B7-H1, a third member of the B7 family, co-stimulates T-cell proliferation and interleukin-10 secretion. Nat Med 1999;5:1365-9.
18. Krambeck AE, Dong H, Thompson RH, Kuntz SM, Lohse CM, Leibovich BC, et al. Survivin and b7-h1 are collaborative predictors of survival and represent potential therapeutic targets for patients with renal cell carcinoma. Clin Cancer Res 2007;13: 1749-56.
19. Pantuck AJ, Zisman A, Belldegrun AS. The changing natural history of renal cell carcinoma, J Urol 2001;166:1611-23.
20. Salceda S, Tang T, Kmet M, Munteanu A, Ghosh M, Macina R, et al. The immunomodulatory protein B7-H4 is overexpressed in breast and ovarian cancers and promotes epithelial cell transformation. Exp Cell Res 2005;306:128-41.
21. Watanabe N, Gavrieli M, Sedy JR, Yang J, Fallarino F, Loftin SK, et al. BTLA is a lymphocyte inhibitory receptor with similarities to CTLA-4 and PD-1. Nat Immunol 2003;4:670-9.
22. Tringler B, Liu W, Corral L, Torkko KC, Enomoto T, Davidson S, et al. B7-H4 overexpression in ovarian tumors. Gynecol Oncol 2006;100:44-52.
23. Bander NH, Finstad CL, Cordon-Cardo C, Ramsawak RD, Vaughan ED Jr, Whitmore WF Jr, et al. Analysis of a mouse monoclonal antibody that reacts with a specific region of the human proximal tubule and subsets renal cell carcinomas. Cancer Res 1989;49:6774-80.
24. van den Berg E, van der Hout AH, Oosterhuis JW, Störkel S, Dijkhuizen T, Dam A, et al. Cytogenetic analysis of epithelial renal-cell tumors: relationship with a new histopathological classification. Int J Cancer 1993;55:223-7.
25. Webster WS, Lohse CM, Thompson RH, Dong H, Frigola X, Dicks DL, et al. Mononuclear cell infiltration in clear-cell renal cell carcinoma independently predicts patient survival. Cancer 2006;

107:46-53.

26. Leppert JT, Pantuck AJ, Figlin RA, Belldegrun AS. The role of molecular markers in the staging of renal cell carcinoma. BJU Int 2007;99:1208-11.

27. Gonzalgo ML, Eisenberger CF, Lee SM, Trock BJ, Marshall FF, Hortopan S, et al. Prognostic significance of preoperative molecular serum analysis in renal cancer. Clin Cancer Res 2002;8: 1878-81.