RESEARCH ARTICLE

DNA Ligase4 as a Prognostic Marker in Nasopharyngeal Cancer Patients Treated with Radiotherapy

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Abstract

Background: The capability for DNA double-strand breaks (DSBs) repair is crucial for inherent radiosensitivity of tumor and normal cells. We have investigated the clinicopathologic significance of DNA repair gene expression in nasopharyngeal (NP) carcinoma. Materials and Methods: A total of 65 NP cancer patients who received radiotherapy were included. The immunopositivity to Ku 70, DNA-PKcs, MRN, RAD50, XRCC4, and LIG4 were examined in all tumor tissues. Results: The patients comprised 42 males and 23 females, with a median age of 56 years (range, 18-84). The expression levels of RAD50 (0,+1,+2,+3) were 27.7%, 32.3%, 21.5%, and 18.5%. LIG4 (±) were 43.1% and 56.9% respectively. The 5-year OS rate of patients with LIG4 (±) were 90% and 67.9%, respectively (p=0.035). The 5-year TTP rate of patients with LIG4 (±) were 75.9%, 55.5%, respectively (P=0.039). Conclusions: Our results suggest the possibility of predicting the radiosensitivity of NP cancer by performing immunohistochemical analysis of LIG4.

Keywords: Nasopharyngeal carcinoma - radiotherapy - nonhomologous end joining - DNA repair genes - LIG4

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Introduction

Nasopharyngeal (NP) carcinoma arises from the lining of the nasopharynx, the narrow passage behind the nasal cavity. Worldwide, there are about 80,000 incident cases and 50,000 deaths annually (Ferlay et al., 2010). The incidence of NP carcinoma demonstrates marked geographical variation. In the United States and Western Europe, it is rare with an incidence of 0.5 to 2 per 100,000 (Parkin et al., 2005; Chang et al., 2006). In contrast, NP carcinoma is endemic in southern China, including Hong Kong, where the incidence may reach 25 cases per 100,000 per year. Intermediate risk regions include Southeast Asia, North Africa and the Middle Ease, and the Arctic. In South Korea, the incidence of NP cancer is 0.7 per 100,000 according to the Korean National Cancer Registry Report 2005, which is lower than that of Hong Kong but still much higher than that of Western countries (Kim et al., 2011).

Because of the anatomical location of the nasopharynx and its proximity to critical neurovascular structures, radiation therapy (RT), rather than surgery, is a mainstay treatment for early stage NP carcinoma. And for more advanced disease, concurrent chemoradiation therapy (CCRT) reduces the rate of distant metastasis and improves local control and overall survival compared to RT alone (Teo et al., 1996; Chua et al., 2002; Kwong et al., 2004; Ma et al., 2008). Thus, radiosensitivity is one of the most important factors determining the prognosis of NP cancer patients. However, methods to predict the radiosensitivity of NP cancer have not yet been developed. Most radiobiologists currently think that radiation primarily causes cell death by double-strand DNA damage, and that the inability of cancer cells to repair such damage with fidelity results in their death (Dikomey et al., 1998).

Ionizing radiation induces DNA double-strand breaks (DSBs), highly cytotoxic and cytostatic forms of damage. The capability for DNA DSBs repair is crucial for inherent radiosensitivity of tumor and normal cells. Repair of DSBs presumably involves two main mechanisms, nonhomologous end joining (NHEJ) and homologous recombination (HR). NHEJ is likely to be the major mechanism of repair of radiation-induced DSBs in humans because it is dominant for the repair of somatic cells and proliferating cells in the G1 stage, whereas HR is important for early embryogenesis and repair of proliferation (Hoeijmakers, 2009; Sakata et al., 2001; Kim et al., 2011). In addition, Ku 70, DNA-PKcs,

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MRN complex, RAD50, XRCC4, and DNA ligase4 (LIG4) play a critical role in DNA DSB repair through NHEJ (Iliakis, 2009; Davis et al., 2013). These DNA repair proteins in tumor cells may be potential prognostic markers for the prediction of the RT outcome. It has been demonstrated that down-regulated expressions of these molecules significantly sensitize cancer cell lines to ionizing radiation. But some studies showed opposite results. In those studies, patients with higher level of DNA repair proteins had better prognosis than patients with lower level.

In this study, we aimed to determine the relationship between the expression of DNA DSBs repair proteins and the prognosis of NP cancer patients treated with RT.

Materials and Methods

Patients and tumors

Data collection was overseen by the institutional review board (DAUH-IRB-14-090). 65 patients who were histologically confirmed nasophayngeal cancer and treated with radiotherapy with or without chemotherapy from April 2000 to March 2009 at Dong-A University Medical Center and Busan National University Hospital were included in this study. For histological diagnosis, specimens were obtained from nasopharyngeal and lymph node biopsy in 61 and 4 patients, respectively. They were staged according to the TNM classification as presented in the AJCC Cancer Staging Manual (Stephen BE, 2010). Clinical follow-up information was obtained by review of the patients' records and retrospectively analyzed.

Immunohistochemistry

Immunohistochemistry was performed automatically using a Ventana autostainer (Benchmark; Ventana Medical Systems, Tucson, Arizona, USA). For antigen retrieval, 4 ~ 6 um-thick tissue sections were treated with retrieval solution (Ventana) and were heated at 100°C for 60 minutes. Endogenous peroxide activity was blocked by immersion in 3% hydrogen peroxide for 4 minutes with diluted primary antibodies for Ku (p70) (1:50, Thermo Fisher Scientific, CA, USA), DNA-PKcs (1:200, Thermo Fisher Scientific, CA, USA), NBN (1:50, Sigma, MO, USA), RAD50 (1:200, Santa Cruz, CA, USA), XRCC4 (1:200, Sigma, MO, USA), and LIG4 (1:50, Sigma, MO, USA); the tissue sections were incubated for 1 hour at 36°C. Immunoperoxidase staining was performed using the DAB system (iView DAB detection kit, Ventana) and the sections were lightly counterstained with hematoxylin. The slides were reviewed by two independent pathologists.

Statistical analysis

Categorical variables were summarized by counts and relative frequencies; continuous variables, by their median and range. The correlation between each markers and categorical variables were estimated by Kruskal-Wallis test; the correlation between each markers and continuous variables by linear regression. The probability of survival was calculated using the Kaplan-Meier method. The log-rank test was used to evaluate the prognostic factors of the survival rate. The OS was calculated from the first day of diagnosis to the date of death from any cause or date of last follow-up. The TTP was calculated from the day of diagnosis to the locoregional and/or distant relapse in completely responded patients or by progression of disease in partially responded patients. Statistical significance was established as P value less than 0.05.

Results

Patient characteristics

The median age of the patients was 56 years (range, 18-84), and the study population included 42 (64.6%) females and 23 (35.4%) males. The stages of patients were I, II (a+b), III, and IV (a+b) in 1, 13, 21, and 30 patients, respectively (Table 1). The median follow-up duration from the date of diagnosis was 40.7 months (range, 1.7-111). The tissue types of tumors were squamous, non-keratinizing, and poorly differentiated types in 31 (47.7%), 15 (23.1%), and 19 (29.2%) patients, respectively. At the end of this study period, 8 (12.3%) patients had died from progression of NP cancer (n=5), sepsis (n=2) and heart failure (n=1).

Treatment delivery and response

We treated all 65 patients with RT. Twenty-eight patients received CCRT alone. 8 patients received RT alone. Ten patients received RT after induction chemotherapy and 6 patients received CCRT after induction chemotherapy. 10 patients were treated with induction chemotherapy, CCRT, and chemotherapy, respectively. Only 3 patients were treated with chemotherapy followed by CCRT. Mean radiation dose was 67.88 cGy (range, 52.50 - 93.80 cGy), and the chemotherapy during CCRT was cisplatin alone

Table 1. Patients' Characteristics

Characteristics (range)	N=65	%
Gender		
Male	42	64.6
Female	23	35.4
Age		
Median (range)	56 (18-84)	
≥60	25	38.5
< 60	40	61.5
Stage		
I	1	1.5
IIa	4	9.2
IIb	9	13.8
III	21	32.3
IVa	26	40
IVb	4	6.2
Tissue type		
Type I: Squamous	31	47.7
Type II: Non-keratinizing	15	23.1
Type III: Poorly differentiated	19	29.2
Treatment		
RT	8	12.3
CCRT	28	43.1
CCRT -> CT	3	4.6
iCT -> RT	10	15.4
iCT -> CCRT	6	9.2
iCT ->CCRT -> CT	10	15.4

*RT: radiotherapy; CCRT: concurrent chemoradiation therapy; CT: chemotherapy; iCT: induction chemotherapy

Table 2. Analysis of Prognostic Factors of Time to Progression and Overall Survival

Characteristics	Time to progression 5 year (%)	P value	Overall survival 5 year (%)	P value
Gender				
Male	66	0.792	78	0.659
Female	63		81.8	
Age				
≥60	68.7	0.905	74.1	0.259
< 60	63.7		82.8	
Stage				
I - III	79.3	0.036	84.8	0.048
IV	23		70.7	
Tissue type				
Squamous	60.1	0.622	72.3	0.328
Non-squamous	77.9		93.4	
LIG4 expression				
LIG4 (+)	75.9	0.037	90	0.035
LIG4 (-)	55.5		67.9	
RAD50 expression				
RAD50 (0-1)	67.2	0.427	83.3	0.216
RAD50 (2-3)	64.4		69.8	

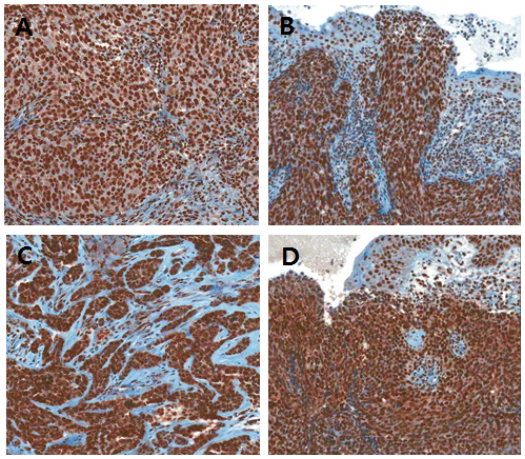

Figure 1. Immunohistochemistry of Head and Neck Cancer A) Ku 70, B; DNA-PKcs, C) NBN, Dl XRCC4, x200. Most of the nuceli of poorly differentiated carcinoma cells express strong immnunoreactivity

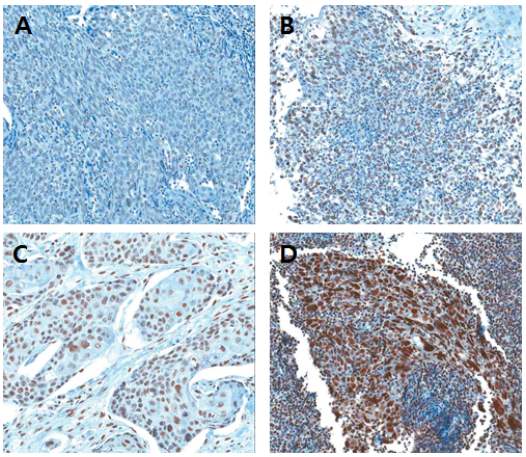

Figure 2. Immunohistochemistry of head and neck cancer (RAD 50, X200 A) negative expression, B) (+1) expression, C) (+2) expression, D) (+3) expression). The cancer cells diversely. Express RAD 50 in nuclei

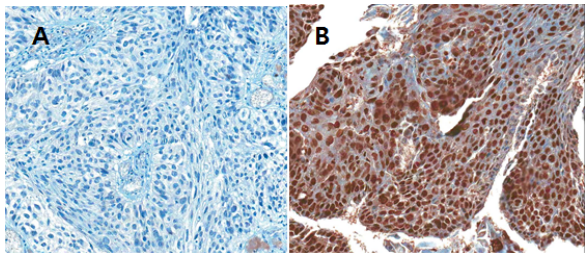

Figure 3. Immunohistochemistry of Head and Neck Cancer (LIG4, X200 A) negative expression, B) positive expression). The cancer cells diversely express LIG4 in nuclei

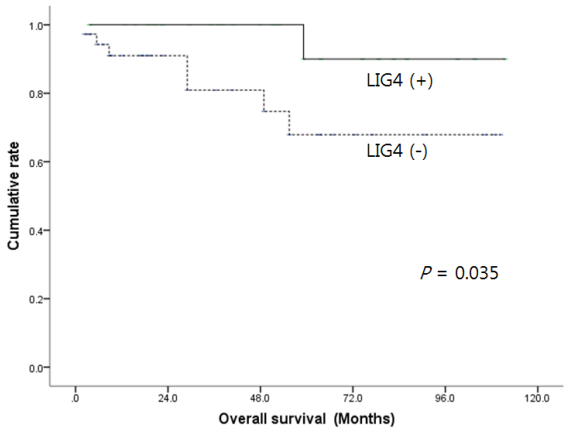

Figure 4. Overall Survival (Kaplan-Meier Estimated Survival Curves) in patients with Nasopharyngeal Carcinomas with Expression of LIG4

(75mg/m^2) delivered every 3 weeks. All the chemotherapy regimens included cisplatin. 29 patients were treated with cisplatin and 5-FU, 23 were cisplan and docetaxel, 1 was cisplatin with S-1, and 4 were cisplatin alone. As a result of treatment, there were complete response (CR) in 33 patients (50.8%), partial response (PR) in 23 patients (35.4%), stable disease in 5 patients (7.7%), and progressive disease in 4 patients (6.2%).

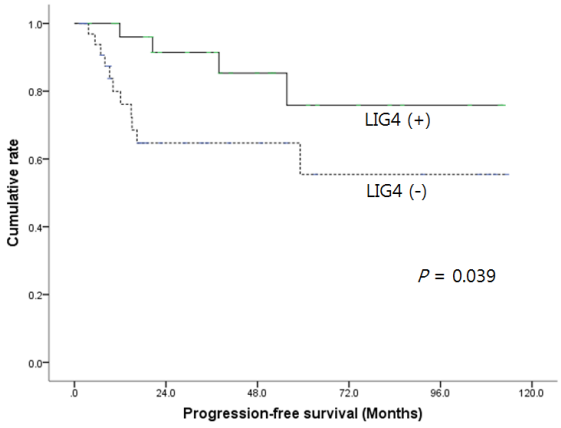

Figure 5. Time to Progression (Kaplan-Meier Estimated Survival Curves) in Patients with Nasopharyngeal Carcinomas with Expression of LIG4

Immunohistochemical staining results

The immunopositivity to Ku 70, DNA-PKcs, MRN, RAD50, XRCC4, and LIG4 were examined in all tumor tissues. There were no apparent differences in the expression of these four proteins between cancerous tissues except RAD50 and LIG4. The expression level of RAD50 (0, +1, +2, +3) were 27.7%, 32.3%, 21.5%, and 18.5%. LIG4 (±) were 43.1%, and 56.9%, respectively.

Correlation between clinical outcome and expression of LIG4 and RAD50

There were no correlations between the positive proportion of LIG4 cells and stage, pathologic type, age, and gender. Also, there was no significant correlation between the expression of LIG4 and CR rate. However, there was a significant correlation between OS and expression of LIG4. The 5-year OS rate of patients with LIG4 (±) were 90% and 67.9%, respectively (p=0.035). There was also a significant correlation between the expression of LIG4 and TTP. The 5-year TTP rate of patients with LIG4 (±) were 75.9%, 55.5%, respectively (p=0.039). Figures 4 and 5 illustrate the Kaplan-Meier estimated survival curves for OS and TTP in patients classified according to the expression level of LIG4. No significant association was found between the level of RAD50 expression and clinical outcome. LIG4 and RAD50 were no statistically significant factor for TTP and OS in multivarient analysis.

Discussion

There are several clinical prognostic factors for NPC such as classification, sex, and age. However, among the same-staged patients, treatment outcomes are variable (Chua et al., 2004). Although there are many other variables that affect the prognosis of NP carcinoma patients, predicting the radiosensitivity of normal and tumor tissues before treatment will provide the most important information to determine the best treatment method for each patient with NP cancer. For that reason, there have been several studies focused on the relationships between expression levels of DNA DSBs repair proteins and prognosis of cancer.

Wilson et al. reported that a higher proportion of Ku70 positive tumor cells is closely associated with poor response to RT and a high risk of locoregional recurrence. The 5-year locoregional control rate was significantly higher in the low Ku70 group (85%) than in the high Ku70 group (42%) (p=0.0042). However, there were no differences in the metastases-free survival rates between the 2 groups (Ku70(+),82%); Ku70(-),78%; p=0.8672) (Lee et al., 2005). Similar results have been reported in tumors at other sites (Komuro et al., 2002; Wilson et al., 2000; Zhao et al., 2000). Uterine cervical cancers with low levels of Ku70 expression in biopsy samples were radiosensitive and displayed a significantly better RT outcome (Wilson et al., 2000).

Bouchaert et al. reported that localized prostate cancer patients with a high tumor level of DNA-PKcs had cancer that was more resistant to RT (p=0.0002). Positive DNA-PKcs nuclear expression was closely associated with biochemical recurrence after RT (p=0.0002). Of the 65 tumors with nuclear DNA-PKcs expression, 34 (>50%) experienced biochemical recurrence. In contrast, only 12 of 67 cases negative for DNA-PKcs had a relapse after treatment (Bouchaert et al., 2012).

In another study, reduced expression of the MRN complex predicted a poor effect of RT in patients with early breast cancer. In this study, moderate/strong expression of the MRN complex was related to a better prognosis compared with negative/weak MRN expression (p=0.02) (Soderlund et al., 2007). Karin et al. explained that their results could be related to the down-regulation or loss of proteins in the MRN complex, impairing the ability to recognize DNA strand breaks and failure to induce DNA damage signaling, cell cycle arrest, and apoptosis, thereby leading to increased risk of relapse. Impaired ability to detect and signal DNA damage is likely to also reduce DNA repair, and this could possibly increase the radiosensitivity of tumor cells. However, there are studies that have had opposite results. MRN complex is a protein complex consisting of Mre11, Rad50 and Nbs1. And overexpression of Nbs1 was a marker of poor prognosis in advanced head-and-neck squamous cell carcinoma and uveal melanoma (Ehlers et al., 2005; Yang et al., 2006). Karin et al. suggested that this discrepancy was due to differences in tumor characteristics and differences in evaluation staining.

Most of these studies tended to associate high NHEJ protein levels with a lower response to RT. However, other studies showed opposite results. In our study, the higher the expression of LIG4, the better prognosis the patients had. Increased expression of LIG4 was a good prognostic marker of disease recurrence or progression and OS in NP cancer patients treated with RT. No significant association was found between the level of RAD50 expression and clinical outcome. These results suggest that the response to DNA damage involving the NHEJ system could be dependent to the types of cancer cells (Pavon et al., 2008). Another possible explanation is that LIG4 may have other functions besides repairing DSB, like the DSB recognition of Nbs1.

Since this is a retrospective study, a number of factors in terms of the patients and tumor characteristics could

not be controlled. Furthermore, this study has some limitations. First, the sample size was too small. Second, the treatment delivery methods were variable, and the sequence of treatment and regimens of chemotherapy were variable. In addition, even though all chemotherapy included cisplatin, we didn't evaluate ERCC1, which is a significant prognostic factor in patients treated with platinum-based chemotherapy. Third, the follow up duration was variable. Consequently, these limitations might cause discrepancies. Nevertheless, this is the first study that suggesting the possibility of forecasting the prognosis of NP cancer treated with RT by performing immunohistochemical analysis of LIG4.

In conclusion, our results suggest the possibility of predicting the intrinsic radiosensitivity of NP cancer by performing immunohistochemical analysis of LIG4. Further prospective studies are needed to confirm our results.

Acknowledgements

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References

Bouchaert P, Guerif S, Debiais C, et al (2012). DNA-PKcs expression predicts response to radiotherapy in prostate cancer. *Int J Radiat Oncol Biol Phys*, **84**, 1179-85.

Chang ET, Adami HO (2006). The enigmatic epidemiology of nasopharyngeal carcinoma. *Cancer Epidemiol Biomarkers Prev*, **15**, 1765-77.

Chua DT, Nicholls JM, Sham JS, et al (2004). Prognostic value of epidermal growth factor receptor expression in patients with advanced stage nasopharyngeal carcinoma treated with induction chemotherapy and radiotherapy. *Int J Radiat Oncol Biol Phys*, **59**, 11-20.

Chua DT, Sham JS, Au GK, et al (2002). Concomitant chemoirradiation for stage III-IV nasopharyngeal carcinoma in Chinese patients: results of a matched cohort analysis. Int *J Radiat Oncol Biol Phys*, **53**, 334-43.

Davis AJ, Chen DJ (2013). DNA double strand break repair via non-homologous end-joining. *Transl Cancer Res*, **2**, 130-43.

Dikomey E, Dahm-Daphi J, Brammer I, et al (1998). Correlation between cellular radiosensitivity and non-repaired double-strand breaks studied in nine mammalian cell lines. *Int J Radiat Biol*, **73**, 269-78.

Ehlers JP, Harbour JW (2005). NBS1 expression as a prognostic marker in uveal melanoma. *Clin Cancer Res*, **11**, 1849-53.

Ferlay J, Shin HR, Bray F, et al (2010). Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. *Int J Cancer*, **127**, 2893-917.

Hoeijmakers JH (2009). DNA damage, aging, and cancer. *N Engl J Med*, **361**, 1475-85.

Iliakis G (2009). Backup pathways of NHEJ in cells of higher eukaryotes: cell cycle dependence. *Radiother Oncol*, **92**, 310-5.

Kim YJ, Go H, Wu HG, et al (2011). Immunohistochemical study identifying prognostic biomolecular markers in nasopharyngeal carcinoma treated by radiotherapy. *Head Neck*, **33**, 1458-66.

Komuro Y, Watanabe T, Hosoi Y, et al (2002). The expression pattern of Ku correlates with tumor radiosensitivity and disease free survival in patients with rectal carcinoma. *Cancer*, **95**, 1199-205.

Kwong DL, Sham JS, Au GK, et al (2004). Concurrent and adjuvant chemotherapy for nasopharyngeal carcinoma: a factorial study. *J Clin Oncol*, **22**, 2643-53.

Lee SW, Cho KJ, Park JH, et al (2005). Expressions of Ku70 and DNA-PKcs as prognostic indicators of local control in nasopharyngeal carcinoma. *Int J Radiat Oncol Biol Phys*, **62**, 1451-7.

Ma BB, Hui EP, Chan AT (2008). Systemic approach to improving treatment outcome in nasopharyngeal carcinoma: current and future directions. *Cancer Sci*, **99**, 1311-8.

Parkin DM, Bray F, Ferlay J, et al (2005). Global cancer statistics, 2002. *CA Cancer J Clin*, **55**, 74-108.

Pavon MA, Parreno M, Leon X, et al (2008). Ku70 predicts response and primary tumor recurrence after therapy in locally advanced head and neck cancer. *Int J Cancer*, **123**, 1068-79.

Sakata K, Matsumoto Y, Tauchi H, et al (2001). Expression of genes involved in repair of DNA double-strand breaks in normal and tumor tissues. *Int J Radiat Oncol Biol Phys*, **49**, 161-7.

Soderlund K, Stal O, Skoog L, et al (2007). Intact Mre11/Rad50/Nbs1 complex predicts good response to radiotherapy in early breast cancer. *Int J Radiat Oncol Biol Phys*, **68**, 50-8.

Stephen BE DR, Carolyn CC, April GF, et al (2010). AJCC cancer staging manual. 7th ed. Philadelphia. edn.

Teo P, Yu P, Lee WY, et al (1996). Significant prognosticators after primary radiotherapy in 903 nondisseminated nasopharyngeal carcinoma evaluated by computer tomography. *Int J Radiat Oncol Biol Phys*, **36**, 291-304.

Wilson CR, Davidson SE, Margison GP, et al (2000). Expression of Ku70 correlates with survival in carcinoma of the cervix. *Br J Cancer*, **83**, 1702-6.

Yang MH, Chiang WC, Chou TY, et al (2006). Increased NBS1 expression is a marker of aggressive head and neck cancer and overexpression of NBS1 contributes to transformation. *Clin Cancer Res*, **12**, 507-15.

Zhao HJ, Hosoi Y, Miyachi H, et al (2000). DNA-dependent protein kinase activity correlates with Ku70 expression and radiation sensitivity in esophageal cancer cell lines. *Clin Cancer Res*, **6**, 1073-8.