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Unfavorable clinical implications of circulating CD44$^+$ lymphocytes in patients with nasopharyngeal carcinoma undergoing radiochemotherapy

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ABSTRACT

Background: To evaluate the use of cellular immunity parameters as predictors of therapy response.
Methods: Circulating lymphocytes were measued by flow cytometry in 94 nasopharyngeal carcinoma (NPC) patients following radiochemotherapy.
Results: Significantly decreased percentage of CD3$^+$, CD4$^+$, and CD8$^+$ lymphocytes, significantly increased proportion of CD44$^+$, CD25$^+$, NK lymphocytes, and an increased CD4$^+$/CD8$^+$ ratio were indicated in NPC patients as compared with healthy controls. Circulating CD44$^+$ lymphocytes in both the N2/N3 and III/IV groups were significantly increased as compared to the N0/N1 and I/II groups, respectively ($P<0.05$). A significant decrease in CD19$^+$ lymphocytes was observed in the III/IV group as compared with the I/II group ($P<0.05$). After radiochemotherapy, NPC patients had significantly ($P<0.05$) decreased percentages of CD4$^+$, CD44$^+$, and CD19$^+$ lymphocytes and a decreased CD4$^+$/CD8$^+$ ratio, whereas the mean percentages of CD8$^+$ and NK lymphocytes were significantly ($P<0.05$) increased. However, compared with the pre-radiochemotherapy values, no significant ($P>0.05$) changes in CD3$^+$ or CD25$^+$ lymphocytes were observed in the NPC-treated group. Follow-up analysis indicated significantly lower DFS for patients with high CD44$^+$ lymphocytes compared to those with low CD44$^+$ lymphocytes after radiochemotherapy.
Conclusion: Circulating CD44$^+$ lymphocytes seems to be a promising criterion to predict survival in NPC patients undergoing radiochemotherapy.

1. Introduction

Nasopharyngeal carcinoma (NPC) is one of the most common malignant tumors in China. The incidence of NPC is higher in parts of southern China, including the provinces of Guangdong, Guangxi, and Hunan. There, the incidence of this disease stands at 20–27.5 per 100,000 people, which is considerably higher than the incidence of <2–3 per 100,000 people in the northern region. However, NPC is the rarest cancer, with an incidence of <1 per 100,000 people among whites in Western countries [1-6]. Radiotherapy (RT) has been the most effective treatment modality for patients with NPC [6]. However, despite impressive progress in diagnosis and treatment, a substantial fraction of the patients with NPC do not respond to conventional treatments (i.e., radiation treatment and/or chemotherapy) and ultimately relapse and die. Intensity-modulated radiotherapy (IMRT) and combined chemotherapy have significantly improved the relapse-free survival of advanced NPC patients [7-10]. To date, however, the prognosis of NPC remains a major problem for oncologists. Because the clinical manifestations of NPC and its biological behavior are unlikely to be detected early and because IMRT treatment at a late stage is not effective in these patients, the 5-year survival rate is only 25%–50% [3, 7, 8]. Clinical staging using Ho's classification has been shown to be invaluable for predicting the outcome of NPC patients after IMRT [11]. However, a fraction of the patients with early-stage disease still develop either locoregional or distant recurrence and die from the disease. Conversely, a proportion of patients with intermediate- or late-stage disease are cured by IMRT [11, 12]. Therefore, additional parameters have been sought to supplement clinical staging for the prognostication of NPC.

T lymphocytes are postulated to play an important role as effector cells responsible for immunologic surveillance, resulting in the elimination of tumor cells from the host. Numerous studies have reported that tumor specific T cells are regularly detected in cancer patients, however these cells have an impaired ability to kill tumors, and these findings have prompted further studies on host immune defense in cancer patients [13-17]. The studies of T cell functions in relation to patient susceptibility to cancer have, however, reported conflicting data regarding immune responses to cancer growth [15-20]. The lymphocytopenic effect of irradiation has been recognized for many years. The various immunologic functions of lymphocytes measured in in

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vitro assays are affected to different extents by radiation. T lymphocyte subpopulations show varying radiosensitivities, ranging from the extreme radiosensitivity of precursor cells to the radioresistance of effector cells. Suppressor cells have been found to be particularly radiosensitive [21–23]. This challenges the immune surveillance theory because of the detrimental effect of radiation on the immune system; however, it is not yet clear whether the lymphocytopenic effect of radiotherapy adversely influences the prognosis of irradiated patients.

T lymphocyte subsets and NK cells are the major forms of cellular immunity. Patients with cancer have impaired immune function, and radiochemotherapy may depress patient immunity, which may be related to tumor progression in such patients. Studying these two types of cells may lead to a better understanding of the function of cellular immunity in cancer patients after radiochemotherapy. CD44 lymphocyte is a receptor for hyaluronic acid and can also interact with other ligands, such as osteopontin, collagens, and matrix metalloproteinases (MMPs). The function of CD44 lymphocyte is controlled by its post-translational modifications [24]. CD44 protein participates in a wide variety of cellular functions including lymphocyte activation, recirculation and homing, hematopoiesis, and tumor metastasis. The establishment of metastasis requires that tumor cells acquire new adhesion and migration properties to emigrate from primary sites and colonize distant organs. CD44 is a cell membrane protein often overexpressed on tumor cells and, being both a cell–cell and cell–extracellular matrix adhesion protein, is well positioned to contribute to this process. Furthermore the interaction of CD44 with other cellular proteins involved in motogenesis and proteolysis is a determinant factor in cell migration and invasion [25, 26]. In this study, we examined the immediate effect of irradiation on T cell subsets distribution and whether T lymphocytes are able to provide prognostic information for irradiated NPC patients.

2. Materials and methods

2.1. NPC patients

All procedures complied with the ethical guidelines for human specimens and use of laboratory of blood collection at Zhejiang Province Cancer Hospital, Zhejiang Cancer Center, China. All patients gave their informed consent. From July 2006 to October 2008, ninety-four newly diagnosed patients with NPC were treated with neoadjuvant chemotherapy plus concomitant chemotherapy and IMRT with adjuvant chemotherapy at the Zhejiang Provincial Cancer Hospital. The patients were identified histopathologically as having NPC by pathologists and staged mainly based on the pathology, the clinical manifestation, and the imaging findings of CT and MRI according to the classification system of the International Union Against Cancer (UICC 2002) [27]. 15 patients were stage I/II and 79 were stage III/IV. Histologically, all patients had World Health Organization nonkeratinizing carcinoma. The ages of the patients ranged from 29 to 74 y, median 48 y. Patients with a Karnofsky score >70, treatment interruption of no more than 5 days, and without previous radiotherapy, distant metastasis, or a second, primary cancer were enrolled. Patients with incomplete treatment, or other serious medical disorders, such as stroke, diabetes, hypertension, heart disease and infectious diseases after radiotherapy, were excluded. Among these patients, 77 were male and 17 were female. All procedures including radiochemotherapy treatment, regular immunological examination and follow-ups regarding response to therapy in this group of patients were performed by the same medical group at the Department of Radiotherapy, Zhejiang Province Cancer Hospital. The time of follow-up was valid until April 30, 2010. At the same time, age- and sex-matched 96 healthy volunteers were randomly selected in Hangzhou city, whom not only were absence of disease and weakness, but had no other diseases including inflammation. Among these volunteers, 78 were male, 18 were female, the ages of the controls ranged from 29 to 74 y, median 48.5 y. All volunteers gave their informed consent.

2.2. Comprehensive treatment of radiochemotherapy

The patients were all treated in the same radiotherapy unit, according to the relevant literature [28, 29]. 94 cases received neoadjuvant chemotherapy plus concomitant and adjuvant chemotherapy. All patients received IMRT, as described previously [30, 31]. The prescription dose was 69.0 Gy–75.9 Gy/30–33 F to gross target volume of nasopharynx (GTVnx), 69.0 Gy-69.9 Gy/30–33 F to planning gross target volume of nasopharynx (PGTVnx), 67.5 Gy–69.9 Gy/30–33 F to positive neck lymph nodes (GTVnd), 60.0 Gy/30–33 F to planning target volume one (PTV1), and 50.9 Gy–54.0 Gy/28–30 F to planning target volume two (PTV2). The patients were all treated in the same radiotherapy unit, at the same time the patients also received same chemotherapy. Neoadjuvant and adjuvant chemotherapy protocols included cisplatin (80 mg/m^2 intravenously in three daily doses), followed by fluorouracil (800 mg/m^2 per day) as a continuous 24-h infusion for 4 days. Neoadjuvant and adjuvant chemotherapy were given every 3 weeks for 2 cycles. The concurrent chemotherapy protocol included cisplatin (DDP) (80 mg/m2 intravenously in 3 daily doses) and was given every 3 weeks for 2 cycles. The course of comprehensive radiochemotherapy treatment takes about 120 days.

2.3. Determination of lymphocyte subpopulations

Blood was collected into heparinized tubes 1 day before the initiation of radiochemotherapy and immediately after the completion of the course of comprehensive radiochemotherapy treatment. In the randomized control group, the blood was collected on a single occasion. One hundred microliters of the heparinized blood was mixed with 20 μl of each of the following fluorescent mouse anti-human monoclonal antibodies according to the reagent instructions: CD3, CD4, CD8, CD19, CD25, CD44 and CD56 (Becton Dickinson, San Jose, CA). The mixtures were incubated for 30 min in a dark room, washed with 3 ml of PBS, and centrifuged at 1500 rpm for 5 min. The supernatant was discarded, and the pellet was resuspended with 1 ml of PBS for flow cytometric analysis. The percentage of fluorescent-positive cells was determined by flow cytometry (Becton Dickinson). Further analysis of the lymphocyte subpopulations was requested from ninety-four patients were asked when the NPC diagnosis was initially confirmed. Among these 94 patients who were evaluated after radiochemotherapy, 31 recipients underwent the dynamic follow-up examinations of their lymphocyte subpopulations 4–5 times over intervals of 3–6 months according to the principle of voluntary participation. The time of lymphocyte subpopulation evaluations is at the day before treatment and the day after treatment at follow-up. The experiments were repeated three times and the mean value was calculated for statistical analysis.

2.4. Statistical analysis

SPSS 15.0 software was used for statistical analysis. Initially, a normal distribution test was performed for measurement data. Normally distributed data were then analyzed using a paired *t*-test, whereas non-normally distributed data were analyzed using the Mann–Whitney *U* test. As a measure of prognosis, we analyzed the clinical data concerning disease-free survival (DFS), defined as the time from the day of the initial flow cytometry examination to first recurrence or last contact. DFS was calculated using the Kaplan–Meier method and Cox regression models. The relationship between $CD44^+$ lymphocytes changes and the outcome of patients was analyzed using the Jonckheere–Terpstra Test. The log-rank test was used for intergroup comparisons. The radiation toxicities were compared using the Wilcoxon–Mann–Whitney test for two samples. All tests were 2-sided, and a $P<0.05$ was considered significant.