Original Article

Human Leukocyte Antigen-A Allele Distribution in Nasopharyngeal Carcinoma Patients Showing Anti-Melanoma-Associated Antigen A or Synovial Sarcoma X-2 T Cell Response in Blood

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Abstract

Background: Development of innovative immunotherapy is imperative to improve the poor survival of the nasopharyngeal carcinoma (NPC) patients. In this study, we evaluated the T cell response to melanoma-associated antigen (MAGE)-A1, MAGE-A3, or synovial sarcoma X-2 (SSX-2) in the peripheral blood of treatment-naive NPC patients. The relationship of responses among the three proteins and the human leukocyte antigen (HLA)-A types were analyzed to provide evidence of designing novel therapy.
Methods: Sixty-one NPC patients admitted into the Tumor Hospital affiliated to the Xinjiang Medical University between March 2015 and July 2016 were enrolled. Mononuclear cells were isolated from the peripheral blood before any treatment. HLA-A alleles were typed with Sanger sequence-based typing technique. The T cell response to the MAGE-A1, MAGE-A3, or SSX-2 was evaluated with the Enzyme-Linked ImmunoSpot assay. Mann-Whitney *U*-test was used to compare the T cell responses from different groups. Spearman's rank correlation was used to analyze the relationship of T cell responses.
Results: HLA-A*02:01, A*02:07, and A*24:02 were the three most frequent alleles (18.9%, 12.3%, and 11.5%, respectively) among the 22 detected alleles. 31.1%, 19.7%, and 16.4% of the patients displayed MAGE-A1, MAGE-A3, or SSX-2-specific T cell response, respectively. The magnitudes of response to the three proteins were 32.5, 38.0, and 28.7 SFC/10^6 peripheral blood mononuclear cells, respectively. The T cell response against the three proteins correlated with each other to different extent. The percentage of A*02:01 and A*24:02 carriers were significantly higher in patients responding to any of the three proteins compared to the nonresponders.
Conclusion: MAGE-A1, MAGE-A3, or SSX-2-specific T cell responses were detectable in a subgroup of NPC patients, the frequency and magnitude of which were correlated.

Key words: Human Leukocyte Antigen-A; Melanoma-Associated Antigen-A; Nasopharyngeal Carcinoma; Synovial Sarcoma X-2; T Cell Response

Introduction

Nasopharyngeal carcinoma (NPC) is a common type of malignant tumor in the head and neck region. The incidence and mortality of NPC have been staying high in China compared to the rest of the world.[1,2] Genetic susceptibility, Epstein-Barr virus (EBV) infection, and exposure to chemical carcinogens are three major

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factors contributing to the initiation and development of NPC.[3,4] Radiation therapy combined with chemotherapy is currently the most commonly used approach to treat NPC. Unfortunately, patients with advanced NPC respond poorly to this treatment modality mainly due to regional recurrence and distant metastases.[5] It is thus imperative to design alternative or adjunctive therapy to improve the prognosis of the patients with NPC. Accumulated knowledge in oncoimmunology allowed the development of innovative antitumor immunotherapy. Harnessing the adaptive immune arm where antigen-specific T cells play the major role may help overcoming the hurdle of treating the advanced NPC.

Human leukocyte antigens (HLAs), the genes coding major histocompatibility complex (MHC) expressing on human cell surface, displayed a great genetic heterogeneity within and among populations. This heterogeneity is the molecular biological factor contributing to distinct immune response of individuals. MHC Class I molecules present peptides derived from endogenous protein onto the surface of antigen-presenting cells whereas MHC Class II molecules is responsible for processing the exogenous antigens.[6] The antigens presented by MHC molecules can be recognized by T cells, thus initiating adaptive immune responses.[7] The differences of MHC molecule amino acid sequence result in distinct host epitope preference and response magnitude to the same antigen. It is reported that different alleles of HLA-A molecules, one of the main components of MHC Class I complex, affect the individual susceptibility to NPC.[8]

Cancer-testis antigens (CTAs) refer to proteins found in tumor tissues with restricted expression in normal testis and placenta. The restricted expression pattern and potent immunogenicity make them suitable immunotherapy targets, which have attracted broad interest.[9,10] Melanoma-associated antigen A (MAGE-A) and synovial sarcoma X (SSX) breakpoint proteins belong to CTAs. There are 12 members of MAGE-A family, MAGE-A1 to -A12. The gene coding MAGE-A proteins clustered on chromosome Xq28.[11] Evidences show that epitopes derived from MAGE-A1 and MAGE-A3 can be presented by the MHC I complex on cell surface. The MHC I complex loaded with MAGE-A-derived epitopes can be recognized by cytotoxic T cells.[12] The cytotoxic T cells can thus induce apoptosis of the cells presenting the epitope in a MHC-restricted way.[13] The research on SSX family as immunotherapy targets came along with the detection of their presence of multiple types of cancers. There are nine members of the SSX gene family, named as SSX-1 to -9.[14] SSX-2 received extra attention because it can be detected in a wide variety of cancers including head and neck cancers.[15] Research showed that both humoral immunity and adaptive immunity could be elicited by SSX-2 in cancer patients.[16,17]

The frequent presence of MAGE-A1, MAGE-A3, and SSX-2 in tumor tissue was reported in cases of ovarian cancer, head and neck squamous cell carcinoma, non-small cell lung carcinoma and prostate cancer, etc.[18-21] In line with this, T cells that can be activated by these proteins were also detected. Cesson *et al*.[22] conducted a research with peripheral blood samples obtained from head and neck cancer patients. They found that several epitopes derived from MAGE-A3 occurred naturally in the patients and could be presented and recognized by specific CD4(+) T cells. It was also reported that novel immunotherapy related to these CTAs was evaluated in clinical trials among non-small cell lung cancer and melanoma.[23-25] However, it remains unclear whether the three CTAs and their specific T cell response can be detected in peripheral blood from patients with advanced NPC. In our research, we tried to address this question using peripheral blood mononuclear cells (PBMCs) from advanced NPC patients through the Enzyme-Linked ImmunoSpot (ELISPOT) assay. We also studied if the antigen-specific T cell response was related to specific HLA-A types and clinical characteristics of the patients. By doing so, we hope to expand our knowledge about immune response elicited by CTAs and improve our understanding in treating NPC.

Methods

Ethical approval

The study was approved by the Institutional Ethics Committee of the Affiliated Tumor Hospital of Xinjiang Medical University. All patients have signed informed consent before the sample was obtained.

Study population

A total of 61 NPC patients admitted into the Tumor Hospital affiliated to the Xinjiang Medical University between March 2015 and July 2016 were recruited in this study. Of all the patients, 45 were male and 16 were female. The age of the patients ranged from 17 to 84 years with median at 48 years. Twelve patients were classified as T Stage 1–2 while 49 patients as Stage 3–4 according to the 7th edition of UICC/AJCC TNM staging system. When referring to the clinical staging system, 33 and 28 patients were grouped as Stage III and IVa [Table 1].

Human leukocyte antigen-A typing

Genomic DNA was extracted with the genomic DNA extraction kit (Tiangen, Beijing, China) from whole blood collected from the patients. The DNA concentration and purity were measured by ultraviolet spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA). DNA with *A*260/*A*280 between 1.8 and 1.9 and concentration between 300 and 500 g/μl were stored in −80°C freezer until HLA-A typed. The HLA-A types were determined with Sanger sequencing-based typing method.

Peripheral blood mononuclear cell

Blood was collected into the Vacutainer containing ethylenediaminetetraacetic acid from median basilica vein of each patient before they were treated. PBMCs were isolated by density gradient separation.[26] The viable cell number was calculated by counting cells after stained with trypan blue. Cells resuspended at 3 mol/ml were used for ELISPOT assay.

Ex vivo interferon-γ Enzyme-Linked ImmunoSpot assay

Three overlapping peptide pools representing the whole amino acid sequence of MAGE-A1, MAGE-A3, and SSX-2 were synthesized according to the consensus subtype sequence found in GenBank (https://www.ncbi.nlm.nih.gov/genbank/). The peptides were synthesized from Sigma (Sigma-Aldrich, Saint Louis, USA). ELISPOT assays were carried out using the kit Human interferon-γ (IFN-gamma) ELISpot BASIC (ALP) from MABTECH (MABTECH, Australia). Briefly, 96-well polyvinylidene fluoride (PVDF) plates (Millipore Corp., Bedford MA, USA) were coated with catch antibody and blocked with Roswell Park Memorial Institute medium (RPMI) containing 10% fetal bovine serum (Sigma-Aldrich, Saint Louis, USA) after 2-h incubation. Sixty thousand PBMCs mixed with one peptide pool were transferred into the coated PVDF plate. Meanwhile, PBMCs treated with phytohemagglutinin (Sigma-Aldrich, Saint Louis, USA) were used as positive control while cells with no treatment were negative controls. After being incubated at 5% CO_2 and 37°C overnight, the plates were washed with PBS (Sigma-Aldrich, Saint Louis, USA) and incubated with detecting antibody at room temperature after the cells were discarded. Two hours later, surplus detection antibody was washed away and Streptavidin-ALP was added. After incubating at room temperature for 45 min, the plate was washed thoroughly with PBS before being developed with AP Conjugate Substrate Kit (Bio-Rad Laboratories Ltd., California, USA). Each spot represents a cell secreting IFN-gamma in response to peptide pulse, which was referred as spot-forming cell (SFC). Adjusted SFC after subtracting average negative values was expressed as SFC/10^6 PBMCs. Responses were considered positive when the quantity of SFC in the well was two times greater than the average value of negative control while the value of negative control was <30 SFC/10^6 PBMCs.[27]

Table 1: General clinical features of 61 NPC patients

Clinical characteristics	*n* (%)
Age	
≥48 years	30 (49.2)
<48 years	31(50.8)
Sex	
Male	45 (73.8)
Female	16 (26.2)
Tumour size (T)	
T1-2	12 (19.7)
T3-4	49 (80.3)
Node (N)	
N0-2	48 (78.7)
N3	13 (21.3)
Metastasis (M)	
No	56 (91.8)
Yes	5 (8.2)
Clinical staging	
III	33 (54.1)
IVa	28 (45.9)
Family history	
No	48 (78.7)
Yes	13 (21.3)

NPC: Nasopharyngealcarcinoma

Statistical analysis

SPSS 21.0 (IBM, Chicago, USA) was used for the data analysis. If the data distributed normally, independent samples *t*-test was used to compare the mean values between two unrelated groups. Rank-sum test was used when the samples display nonnormal distribution. Countable variables, such as gender, sex, and HLA-A types, were summed up as percentage. Differences between two groups were tested with Chi-square test or 2 × 2 contingency table. Mann-Whitney *U*-test was used to compare the T cell responses from different groups. Spearman's rank correlation was used to analyze the relationship of T cell responses. A $P < 0.05$ was considered statistically significant.

Results

Human leukocyte antigen-A allele distribution in nasopharyngeal carcinoma patients

The general clinical features of 61 NPC patients can be found in Table 1. Twenty-two HLA-A alleles were detected in 61 NPC patients. The three most common alleles found in our cohort were HLA-A*02:01 (18.9%), A*02:07 (12.3%), and A*24:02 (11.5%) [Figure 1].

Frequency and magnitude of melanoma-associated antigen-A1, melanoma-associated antigen-A3, or synovial sarcoma X-2-specific T cell response in nasopharyngeal carcinoma patients

IFN-gamma ELISpot assay was employed to determine the MAGE-A1, MAGE-A3, or SSX-2-specific T cell response in peripheral blood of the NPC patients. Of 61 patients, T cell responses to MAGE-A1, MAGE-A3, and SSX-2 peptide pools were detected in 31.1%, 19.7%, and 16.4% of the patients, respectively. Within the patients displaying T cell response, the average response magnitudes to MAGE-A1, MAGE-A3, or SSX-2 were 32.5, 38.0, and 28.7 SFC/10^6 PBMCs, respectively [Figure 2].

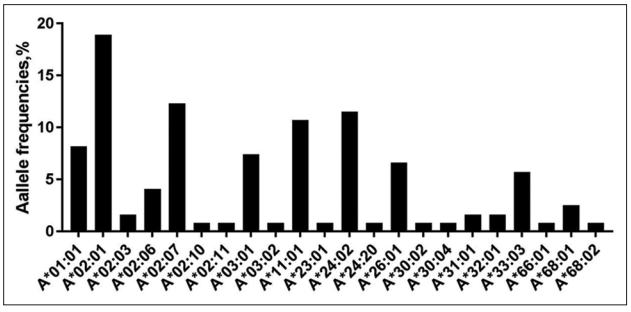

Figure 1: HLA-A allele frequencies in 61 NPC patients. Twenty-two HLA-A alleles were detected in 61 NPC patients. The three most common alleles found in our cohort were HLA-A*02:01 (18.9%), A*02:07 (12.3%), and A*24:02 (11.5%). HLA: Human leukocyte antigen; NPC: Nasopharyngeal carcinoma.

In the subgroup of patients from TNM Stage T3-4, the magnitude of T cell response was significantly higher than patients from T1-2 (11.71 vs. 5.20 SFC/10^6 PBMCs, $Z = -2.230$, $P = 0.026$). When grouping the patients according to the clinical staging system, patients from Stage III showed significantly elevated magnitude of T cell response to MAGE-A3 compared to those from Stage IVa (8.21 vs. 4.10 SFC/10^6 PBMCs, $Z = -1.966$, $P = 0.049$). It was noted that on average, magnitude of the response to SSX-2 in patients aged over 48 years was significantly stronger than those aged 48 years or younger (7.33 vs. 2.75 SFC/10^6 PBMCs, $Z = -2.412$, $P = 0.016$).

Correlations between T cell response to melanoma-associated antigen-A1, melanoma-associated antigen-A3, and synovial sarcoma X-2

To see whether one patient could have shared immune response against any two of MAGE-A1, MAGE-A3, and SSX2, we analyzed the correlation of the response frequency or magnitude between two CTAs. The frequency and magnitude of MAGE-A1-specific T cell response were both mildly associated with MAGE-A3-specific response ($r = 0.380$, $P = 0.003$ for frequency; $r = 0.475$, $P = 0.000$ for magnitude). The frequency of MAGE-A1-specific response was also mildly correlated with that of SSX-2 ($r = 0.372$, $P = 0.003$), but the magnitude was intermediately correlated between the two ($r = 0.653$, $P = 0.000$). The correlations of the T cell response were also observed between MAGE-A3 and SSX-2, the frequency and magnitude of which were mildly and intermediately associated, respectively ($r = 0.449$, $P = 0.000$ for frequency; $r = 0.617$, $P = 0.000$ for magnitude) [Table 2 and Figure 3].

Human leukocyte antigen-A allele distribution in patients showing melanoma-associated antigen-A1, melanoma-associated antigen-A3, or synovial sarcoma X-2-specific T cell response

Fourteen HLA-A alleles were detected in patients displaying MAGE-A1, MAGE-A3, or SSX-2-specific T cell reactivity. However, the HLA-A alleles distribute differentially in these patients. HLA-A*02:01 and A*24:02 were the two alleles most frequently observed. To be more specific, HLA-A*02:01 was seen in 21.1%, 20.8%, and 25.0% of the patients responding to MAGE-A1, MAGE-A3, or SSX-2, respectively. HLA-A*24:02 was detected in 18.4%, 25.0%, and 25.0% of the patients responding to MAGE-A1, MAGE-A3, and SSX-2, respectively [Figure 4].

Table 2: Correlation of the T cell responses to MAGE-A1, MAGE-A3, and SSX-2 peptide pools in peripheral blood of NPC patients

MAGE-A1	MAGE-A3, *n*		SSX-2, *n*	
	Positive	Negative	Positive	Negative
Positive ($n = 19$)	8	11	7	12
Negative ($n = 42$)	4	38	3	39

MAGE-A: Melanoma-associated antigen A; SSX-2: Synovial sarcoma X-2; NPC: Nasopharyngeal carcinoma.

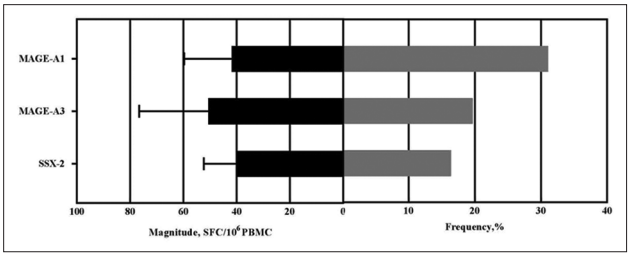

Figure 2: The MAGE-A1, MAGE-A3, and SSX-2-specific T cell response in peripheral blood of 61 NPC patients. IFN-gamma ELISpot assay was employed to determine the MAGE-A1, MAGE-A3, or SSX-2-specific T cell response in peripheral blood of the NPC patients. Of 61 patients, T cell responses to MAGE-A1, MAGE-A3, and SSX-2 peptide pools were detected in 31.1%, 19.7%, and 16.4% of the patients, respectively; within the patients displaying T cell response, the average response magnitudes to MAGE-A1, MAGE-A3, or SSX-2 were 32.5, 38.0, and 28.7 SFC/10^6 PBMCs, respectively. MAGE: Melanoma-associated antigen; SSX-2: Synovial sarcoma X-2; NPC: Nasopharyngeal carcinoma; PBMC: Peripheral blood mononuclear cell; SFC: Spot-forming cell.

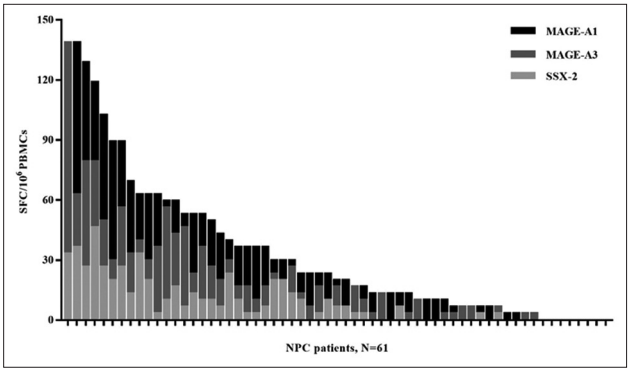

Figure 3: The correlation of T cell response against MAGE-A1, MAGE-A3, and SSX-2 in 61 NPC patients. MAGE: Melanoma-associated antigen; SSX-2: Synovial sarcoma X-2; NPC: Nasopharyngeal carcinoma; PBMC: Peripheral blood mononuclear cell; SFC: Spot-forming cell.

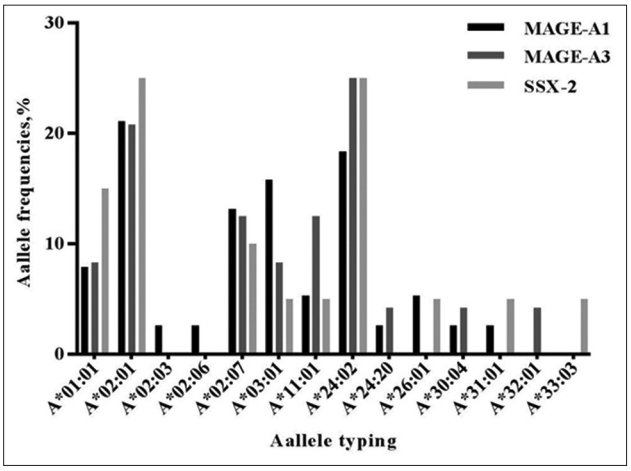

Figure 4: The HLA-A distribution in 61 NPC patients with MAGE-A1, MAGE-A3, or SSX-2-specific T cell response. Fourteen HLA-A alleles were detected in patients displaying MAGE-A1, MAGE-A3, or SSX-2-specific T cell reactivity. However, the HLA-A alleles distribute differentially in these patients. HLA-A*02:01 and A*24:02 were the two alleles most frequently observed. HLA-A: Human leukocyte antigen-A; MAGE: Melanoma-associated antigen; SSX-2: Synovial sarcoma X-2; NPC: Nasopharyngeal carcinoma; PBMC: Peripheral blood mononuclear cell.

Discussion

NPC is one of the common malignant tumors in China. The incidence of NPC is higher in South China.[1] Those who had family history of NPC have higher susceptibility. It is well recognized that genetic predisposition factors play critical roles in the initiation and development of NPC.[2,28] The HLA gene family, clustering on chromosome 6, is the most polymorphic genes in human genome. They encode MHC proteins which present antigens on the cell surface. MHC proteins are mainly comprised Class I molecules such as HLA-A, HLA-B, and HLA-C and Class II molecules such as DRB1, DQA1, DQB1, and DPB1. HLA genes are actively involved in immune surveillance and immune homeostasis, the failure of which is closely related to tumor growth.[29] A study conducted with 8333 people living in Zhejiang, a city in South China, showed that the most frequent alleles were A*11:01, A*24:02, and A*02:01.[30] Studies have consistently identified an association between NPC and HLA-A*11:01, HLA-A*02:07, and HLA-B*58:01.[31] In contrast, Tian *et al*.[32] conducted a study involving 930 NPC patients and revealed that A*02:07 was the most frequently found allele in the cohort. Previous study from our laboratory also reported that the presence of HLA-A*02:06 and B*38:02 as well as the haplotype A*02:07–B*46:01 was related to the risk of NPC.[33,34] The different distributions of alleles among general population and NPC patients indicated that certain HLA alleles might contribute to the susceptibility to NPC.[35]

In this study, we genotyped HLA-A alleles of 61 advanced NPC patients and found that HLA-A*02:01, A*02:07, A*11:01, and A*24:02 were the most frequent alleles among the 22 alleles detected. Our result is consistent with the study conducted by Tian *et al*.[32] among NPC patients.

Radiotherapy is the primary choice in treating NPC, but the survival of the patients with advanced NPC is still to be improved. One option is using the antigen-specific immunotherapy. The general rule of antigen-specific immunotherapy is that the expression of antigen targeted is restricted in malignant tumor, but little in healthy normal tissues. If not, autoimmune diseases can be a serious side effect. MAGE-A1, MAGE-A3, and SSX-2, three well-studied CTAs, meet the requirement and are gaining increasing interest as potential target of developing immunotherapy.[36,37] Although T cell response against MAGE-A1, MAGE-A3, and SSX-2 was found in a wide variety of cancer, such as glioma, melanoma, pancreatic adenocarcinoma, and hepatocellular carcinoma,[38-42] it remained to be defined whether similar response can be observed in advanced NPC.

In this study, we employed *ex vivo* interferon-γ ELISPOT assay to see if there existed any T cell response against MAGE-A1, MAGE-A3, and SSX-2 in peripheral blood of advanced NPC patients. In terms of the average magnitude, MAGE-A1 and MAGE-A3 displayed distinct patterns. More patients in Stage T3-4 displayed MAGE-A1 T cell reactivity than those in Stage T1-2. In contrast, patients in Stage III responded to MAGE-A3 in greater magnitude than those from Stage IVa. The other point worth to mention is that the increased magnitude of SSX-2-specific T cell response was observed in patients older than 48 years. With our current results, however, it is too early to conclude whether the presence of CTA-specific T cell is associated with good or poor prognosis. The question is that whether those T cells were robust enough or too exhausted to suppress the development of cancer. Phenotype characterization and functional study of the epitope-specific T cells might address this problem.

Our study has reported the detection of simultaneous T cell response to several CTAs in the same patient. We discovered in certain NPC patients that the T cell responses to MAGE-A1, MAGE-A3, and SSX-2 were correlated to different extents. The merit of this discovery was the potential existence of epitopes shared by the three tumor antigens. Epitope mapping can be performed in the future to identify the specific peptides of interests.

Antigens presented by MHC Class I can initiate cytotoxic T lymphocyte (CTL) response. A research proved that MAGE-A1 and MAGE-A3 could be processed and presented by MHC Class I molecules to initiate killing from CTL.[43] On the other hand, a research demonstrated that HLA-A2-restricted epitope from the SSX-2 was identified and might activate CD8(+) T in prostate cancer patients.[44] In our study, we compared the HLA-A allele distribution of 61 patients and the patients having MAGE-A1, MAGE-A3, or SSX-2-specific T cell reactivity. It was found that the ratios of HLA-A*02:01 and A*24:02 carriers were higher in the latter group. One explanation can be that certain epitopes of the three CTAs were preferably presented by the MHC complex coded by A*02:01 or A*24:02. To test this hypothesis, the HLA restriction can be characterized after identifying the specific epitopes in these patients. If the epitope happens to be a dominant one, a broad-spectrum immunotherapy can be designed and tested.

In conclusion, MAGE-A1, MAGE-A3, or SSX-2-specific T cell response can be detected in the peripheral blood obtained from NPC patients. It is worthwhile to further study and uncover novel epitopes as target of immune therapy to treat advanced NPC.

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Conflicts of interest

There are no conflicts of interest.

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中晚期鼻咽癌患者外周血中HLA-A基因限制性MAGE-A1、MAGE-A3及SSX-2蛋白诱导的T细胞免疫反应初探

摘要

背景：局部复发和远处转移是鼻咽癌（nasopharyngeal carcinoma, NPC）治疗失败的主要原因，严重影响患者的生存，寻找新的免疫治疗方案意义重大。本研究探讨中晚期NPC初治患者外周血中HLA-A限制性MAGE-A1、A3及SSX-2抗原肽细胞毒性T细胞(CTL)免疫反应水平，并探讨三者之间的内在联系，为中晚期NPC患者细胞免疫治疗提供实验依据。

方法：选择2015年3月～2016年7月新疆医科大学附属肿瘤医院由病理明确诊断的初治中晚期NPC患者61例，采用SBT-sanger法检测患者HLA-A等位基因单倍体分型；采用酶联免疫斑点法(ELISPOT)检测经MAGE-A1、SSX-2抗原肽刺激后的PBMC分泌IFN-γ的CTL细胞的频率及强度。采用Mann Whitney U秩和检验分析患者组间MAGE-A1、MAGE-A3、SSX-2反应强度差异，用Spearman's rank correlation进行相关性分析。

结果：61例患者共检测出HLA-A等位基因分型22种，其中基因频率前三位的分别是A*02:01（18.9%），A*02:07（12.3%）以及A*24:02（11.5%）。鼻咽癌患者外周血中MAGE-A1、MAGE-A3及SSX-2抗原特异性CTL免疫应答阳性率分别为31.1%，19.7%和16.4%；平均反应强度分别为32.5，38.0及28.7 SFC/10^6 PBMC。MAGE-A1、MAGE-A3与SSX-2之间CTL反应频率与强度均成正相关。MAGE-A1、MAGE-A3及SSX-2特异性抗原肽阳性反应患者中携带A*02:01（21.1%, 20.8%和25.0%）和A*24:02（18.4%, 25.0%和25.0%）等位基因型的频率较高。

结论：中晚期NPC患者可诱导HLA-A限制性MAGE-A1、MAGE-A3与SSX-2抗原特异性T细胞免疫反应，且彼此间频率与强度具有一定关联性，可能为中晚期NPC患者免疫治疗的临床应用提供实验室依据。