and outcome of macrophage-mediated cross-presentation should be clarified in the future.

CONCLUSION

The present study demonstrated that selective vaccine delivery to medullary macrophages localized in the medulla, the core of lymph nodes, can be achieved by using an immunologically stealth nanoparticulate delivery system. We also revealed that medullary macrophages have a potential to effectively induce specific $CD8^+$ T cell response to vaccines. These two key findings build a theoretical basis to design a macrophage-oriented cancer vaccine with high potency, which may overcome the limited clinical efficacy of existing cancer vaccines. This strategy may improve the therapeutic efficacy of vaccines against not only cancers but also infectious diseases reactive to T cell immunity, and may thus have great impact in the field of immunotherapy. Cancer vaccines utilizing the CHP nanogel used in the present study have already been tested in clinical trials and confirmed to be highly safe and immunogenic, also supporting the clinical application of macrophage-oriented vaccines utilizing this nanoparticulate delivery system.

MATERIALS AND METHODS

Fabrication and Analysis of the Complex of CHP Nanogel with LPA. CHP, rhodamine-labeled CHP, CHP-NH_2, and CHG were synthesized as described previously.[35−37] LPAs were chemically synthesized by Bio-Synthesis, Inc. (Lewisville, TX). The sequences of the LPAs used were as follows: human MAGE-A4-derived LPA (GSNPARYEFLWGPRALAETSYVKVLEHVVRVNARVRIAYP), mERK2-derived LPA (NDHIAYFLYQILRGLQYIHSANVLHRDLKPSNLLLNT), and human NMW LPA (**SLLMWITQC**YYYYYY**NYKRCFPVI**YYYYYY**CMTWNQMNL**). The MAGE-A4 LPA and mERK2 LPA contain epitopes for murine $CD8^+$ T cells (underlined), and the human NMW LPA does for HLA-A2-restricted NY-ESO-1, HLA-A24-restricted MAGE-A4, and HLA-A24-restricted WT1 epitopes (bold). For analysis of antigen incorporation, these LPAs were labeled with fluorescein amidite (FAM). LPA and CHP were dissolved in dimethyl sulfoxide and phosphate-buffered saline (PBS) containing 6 M urea, respectively. Both solutions were combined and gently mixed at room temperature, followed by dialysis against PBS to remove urea. The resulting solution of the CHP:LPA complex was stored at 4 °C until use. The peptide concentration in the CHP:LPA complex solution was determined by measuring the absorbance at 280 nm, where CHP does not contribute to absorbance. The final concentration of CHP was approximately 10 mg/mL and that of LPA was 0.2−0.4 mg/mL. Complexation of the CHP nanogel and FAM-labeled LPA was confirmed by means of high performance size exclusion chromatography (HPSEC) using a Superose 12 10/300 GL column (GE Healthcare). An aliquot of the sample was injected into the HPSEC system (Shimadzu), eluted with PBS, and detected by means of ultraviolet absorbance at 495 nm. To determine the size of the CHP nanogel and CHP:LPA complex, dynamic light scattering measurement was performed using a Zetasizer Nano ZS (Malvern Instruments, Ltd.) at 633 nm and a 173° detection angle at 25 °C. The measured autocorrelation function was analyzed using the cumulant method. The hydrodynamic diameter (D_H) of the samples was calculated from the Stokes−Einstein equation. The ζ-potential of CHP:LPA complex was also measured using the Zetasizer Nano ZS at a 90° detection angle at 25 °C in PBS. The mERK2 CHP:LPA complex was also analyzed using transmission electron microscopy (TEM). Briefly, the sample was applied to a carbon-coated grid, and the grid was stained with TI blue (Nisshin EM, Japan), dried, and subjected to TEM (HT7700, Hitachi) at an accelerating voltage of 100 kV.

Mice and Tumors. Female BALB/c mice were obtained from SLC Japan and used at 6−12 weeks of age. DUC18 mice, transgenic for TCRα/β that interacts with a K^d-restricted $mERK2_{136-144}$ peptide epitope, were established as described previously.[25] CD90.1-congenic BALB/c mice were kindly provided by Dr. Sakaguchi of Osaka University, Japan. We mated DUC18 mice and CD90.1-congenic mice at our animal facility, and obtained DUC18/CD90.1 mice. T cells isolated from these mice can be traced in *in vitro* and *in vivo* experiments using anti-CD90.1 antibody. All mice were maintained at the Experimental Animal Facility of Mie University. The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Mie University.

CT26 is a colon epithelial tumor cell line that was produced by intrarectally injecting *N*-nitroso-*N*-methylurethane in BALB/c mice.[30] CT26 cells stably expressing human cancer/testis antigen MAGE-A4 (CT26/MAGE-A4) were established as described previously.[21] CMS5a is a subclone derived from CMS5, a 3-methylcholanthrene-induced sarcoma cell line of BALB/c origin, and expresses mERK2 as a neoantigen.[20] In *in vivo* tumor growth experiments, mice (n = 4 or more) were inoculated subcutaneously in the right hind flank with 10^6 CT26/MAGE-A4 or CMS5a cells and monitored three times a week. The tumor size was estimated using the following formula: tumor size (mm^3) = 1/2[length (mm) × width (mm) ^{2}].

Tracking of the Subcutaneously Injected CHP or CHP:LPA Complex. For tracking of the injected CHP nanogel, rhodamine-labeled CHP nanogel was subcutaneously injected to the back of BALB/c mice. Six hours later, the skin harboring DLN was harvested and observed using confocal laser scanning microscope (LSM780, Carl Zeiss, Germany). For tracking of the injected CHP:LPA, a fluorescently labeled LPA complexed with the CHP nanogel was subcutaneously injected into the right hind flank of the mice. The inguinal DLN was harvested 16 h after the injection, mashed, and filtered through a nylon mesh. The resulting cell suspension was analyzed using flow cytometry for incorporation of labeled LPA and expression of CD11b, CD11c, CD169, F4/80, CD3ε, and B220. For immunohistochemical analysis, cryosections were prepared from the DLN. Optimum cutting temperature (O.C.T.) compound-embedded cryosections were stained with fluorescent dye-conjugated anti-CD169 or anti-F4/80 monoclonal antibodies (mAbs) and examined under a fluorescence microscope (BX53F, Olympus).

Immunization of Mice. The CHP:LPA complex or LPA emulsified with IFA (Sigma-Aldrich) was subcutaneously injected into the back of mice at the dose of 50 μg as LPA. Phosphorothioate-containing CpG ODN 1668 (Hokkaido System Science, Japan) or poly-ICLC RNA (Oncovir, Inc.) was simultaneously and subcutaneously injected near the site of vaccination. To deplete macrophages, a clodronate liposome solution (FormuMax Scientific) was subcutaneously injected into the footpad of mice 6 days prior to immunization.

Flow Cytometric Analysis. Fluorescent dye-conjugated mAbs including anti-CD8 (53−6.7), anti-CD4 (RM4−5), anti-interferon (IFN)-γ (XMG1.2), anti-CD80 (2D10.4), anti-CD86 (IT2.2), anti-CD11b (M1/70), anti-CD11c (N418), anti-F4/80 (BM8), anti-CD3ε (145−2C11), anti-CD45R/B220 (RA3−6B2), and anti-CD169 (3D6.112) were purchased from Biolegend. The cell suspension prepared from the inguinal DLN or spleen was stained for surface markers using antibodies at the appropriate concentrations in PBS containing 2% fetal bovine serum for 15 min at 4 °C, and analyzed on a FACSCanto II system (BD Biosciences). For intracellular cytokine staining, splenocytes were incubated with mERK2 LPA or MAGE-A4 LPA for 1 h at 37 °C and then incubated for an additional 6 h with GolgiPlug (BD Bioscience). After permeabilization and fixation using the

Cytofix/Cytoperm Kit (BD Bioscience), the cells were stained with allophycocyanin-conjugated anti-IFN-γ mAb and analyzed using flow cytometry.

T Cell Proliferation and IFN-γ Release Assay. For *in vitro* T cell proliferation assay, the CHP:mERK2 LPA and CpG ODN were subcutaneously injected into the footpad of mice. DLN was resected 20 h after the injection. To isolate $CD11b^+$ cells, the total cell suspension prepared from the DLN was mixed with anti-CD11b microbeads (Miltenyi Biotec) and separated by positive selection on a magnetic bead column. For further fractionation of $CD11b^+$ cells, the cells were stained with FAM-labeled anti-CD11b, PE-labeled anti-CD169, PerCP-Cy5.5-labeled anti-F4/80, and allophycocyanin-labeled anti-CD11c mAbs, and sorted using a FACSAria system (BD Biosciences). The isolated cells (0.1, 0.3, or 1×10^5) were then cocultured with 2.5×10^5 DUC18 T cells prelabeled with carboxyfluorescein succinimidyl ester (CFSE) for 48 h. The dilution of CFSE was measured using flow cytometry to determine cell proliferation. IFN-γ release into the culture medium at 48 h was quantified using a mouse IFN-γ ELISA kit (BD Bioscience).

For *in vivo* T cell proliferation assay, either the CHP:mERK2 LPA or IFA:mERK2 LPA was subcutaneously injected into the back of mice. At day 0 or day 4, 2.5×10^5 DUC18 T cells prelabeled with CFSE were intravenously infused into the vaccinated mice. DLNs were collected on day 3 or day 7, and dilution of CFSE was measured using flow cytometry to determine the *in vivo* proliferation of DUC18 T cells.

***In Vitro* Cross-Presentation by Human Macrophages.** Peripheral blood mononuclear cells were isolated from buffy coats prepared using density gradient centrifugation of the blood of HLA-A0201-positive healthy donors over Ficoll. $CD14^+$ cells were purified with anti-CD14 microbeads and then incubated with GM-CSF, 20 ng/mL in the X-VIVO15 medium, for 7 days to induce differentiation to macrophages. The obtained macrophages were pulsed with human CHP:NMW LPA (10 μg/mL) and stimulated with poly-ICLC RNA (10 μg/mL). The human IFN-γ enzyme-linked immunospot (ELISPOT) assay was performed as previously described. Briefly, a 96-well nitrocellulose ELISPOT plate (Millipore) was coated with an anti-human IFN-γ mAb (clone 1-D1K, Mabtech) overnight at 4 °C. The wells were washed with PBS containing 0.01% Tween 20 (PBS-T) and blocked with the RPMI1640 medium containing 10% fetal calf serum for 2 h at 37 °C. HLA-A0201-restricted NY-ESO-1 epitope-specific $CD8^+$ T cells (clone 1G4, 10^5 cells per well) and antigen-pulsed macrophages were seeded into each well. After incubation for 22 h at 37 °C in 5% CO_2, the plate was washed thoroughly with PBS-T. A biotin-conjugated anti-human IFN-γ mAb (clone 7-B6-1, Mabtech) was then added (final concentration 1.25 μg/mL), and the plate was incubated overnight at 4 °C. After a wash step with PBS-T, a streptavidin-alkaline phosphatase conjugate (1 μg/mL; Roche Diagnostics) was added. After incubation for 60 min at room temperature, the wells were washed thrice with PBS-T and stained using an alkaline phosphatase conjugate substrate kit (Life Technologies). The reaction was stopped by intensive washing with distilled water. After the plate dried out, the number of spots in each well was counted using an ELISPOT reader (Cellular Technologies, Ltd.).

Statistical Analysis. The data were analyzed using Student's *t* test, Dunnett's multiple comparison test, or Tukey-Kramer multiple comparison test. Differences with $p < 0.05$ were considered statistically significant.

Conflict of Interest: The authors declare the following competing financial interest(s): Naozumi Harada is an employee of ImmunoFrontier, Inc. The other authors have no conflicts of interest.

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Supporting Information Available: Characterization of the nanogel:LPA complex, associated *in vivo* mouse immunological data, and *in vitro* experiment with human macrophages. This material is available free of charge *via* the Internet at http://pubs.acs.org.

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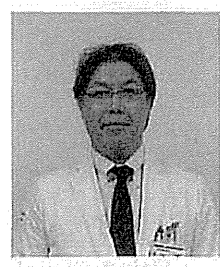
医療

難治性腫瘍の治療成績向上を目指した外科手術を基軸とする集学的治療戦略

大学院医学研究科
（医学部医学科）

教授 平野（ひらの） 聡（さとし）



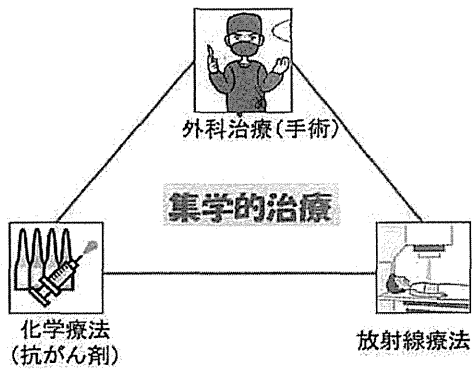
専門分野 ： 消化器外科（胆道・膵臓・上部消化管）
研究のキーワード ： 外科, 放射線治療, ワクチン
HP アドレス ： http://surg2-hokudai.jp/

出身高校：函館ラ・サール高校（北海道）
最終学歴：北海道大学大学院医学研究科

何を目指しているのですか？

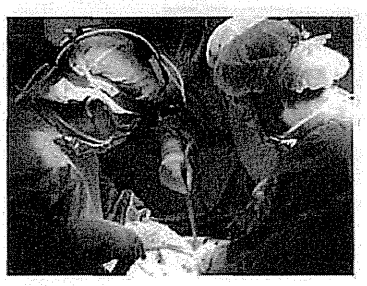
大学院医学研究科消化器外科学分野 II では上部消化管・胆道・膵臓の癌を代表とする悪性疾患やその他の腫瘍性・非腫瘍性疾患を対象として手術治療や臨床・基礎研究を行っています。

当科では超進行癌に対する積極的な拡大根治手術、低悪性度病変に対する適切な縮小手術、早期癌に対する低侵襲内視鏡手術を治療戦略として手術を行っています。その中でも食道癌、胆嚢癌、膵癌はとりわけ悪性度が高く再発しやすいため、**外科**治療をもってしても治療が困難な癌、いわゆる難治癌と呼ばれています。これら難治性で高度に進行した癌の治療においては手術治療だけでは不足であり、術前・術後の手術以外の治療（補助療法）、すなわち抗がん剤による化学療法や**放射線治療**を組み合わせた「集学的治療法」の開発が必要になります。



難治性高度進行癌の治療においては手術治療だけでは不足であり、化学療法や放射線治療と組み合わせた「集学的治療法」の開発が必要になります。

この「集学的治療法」の開発のために私たちが進めているプロジェクトの一つに膵癌術前化学療法の臨床試験があります。術前に化学療法を加えることで術後の癌再発率を下げ、治療成績を向上させるのが目的です。このプロジェクトの特徴は道内にある三つの医育大学の垣根を取り払い、全道各地の 38 施設の内科・外科が共同してチームを組み、文字通り“オール北海道”で大規模な臨床試験を行うというものです。また、診断時に手術適応のない高度進行膵癌・胆道癌の患者さんに対しても新たなプロジェクトがあります。抗癌剤治療や放射線療法が半年以上効果的で病勢の悪化が認められない場合には、通常行わない肝動脈などの血管合併切除再建を伴う積極的な手術治療、すなわち拡大切除術を行うことで良好な成績が得られる可能性があることを世界で初めて報告し、まもなく全国的な多施設共同試験を開始します。これら



高度進行癌に対する化学療法後の手術の様子

の他、手術治療、化学療法、放射線療法など標準的治療法が効かなくなった高度進行あるいは再発癌の患者さんを対象として、副作用の少ない体に優しい補助療法を開発するための癌免疫療法の臨床研究、また最新の分子生物学的な手法を応用した遺伝子治療や分子標的治療の基礎研究にも着手しています。次項では当科の研究実績の中から癌免疫治療の臨床研究を紹介します。

何が課題となっていますか？

癌細胞の表面には癌細胞のみに発現し正常組織には発現しない腫瘍抗原という目印が存在します。当科では多くの癌腫に発現する腫瘍抗原を標的として、「HER2 蛋白 CHP 複合体パルス樹状細胞を用いた癌**ワクチン**第Ⅰ相臨床試験」、「NY-ESO-1 癌抗原蛋白／CpG／リポソーム複合体を用いた癌ワクチン第Ⅰ相臨床試験」など、免疫治療の臨床研究を 2005 年より展開してきました。もともと生体に備わった免疫力を増強させることで抗腫瘍活性を高めることを目的とした、患者さんにとって負担の少ない新しい補助治療です。高度進行・再発癌の患者さんを対象としたこれまでの臨床試験で明らかになったことは、癌免疫治療により高頻度に特異的な免疫反応が誘導されること、また、免疫治療のみで比較的長期間、病状の進行を停滞させることができる症例が存在するという事実です。


実験室で行う“基礎研究”の様子

このように癌ワクチンによって生体内で腫瘍に特異的な免疫反応が誘導される事がわかってきましたが、現在までのところワクチン単独では確実な臨床効果が得られるまでには至っていません。この理由として、腫瘍の免疫逃避機構や免疫抑制性細胞の存在などが知られています。今後はこのような癌ワクチンへの反応良好例を判別するバイオマーカーの開発によりワクチン療法が適応となる症例を選択することや、より強力な免疫反応を誘導できる補助療法の開発と検証が課題と考えています。

次に何を目指しますか？

実験室内での特殊な条件下で得られたデータのみでは臨床現場で使用したり、患者さんに有効な治療にまで発展させることは困難です。実験室で行う“基礎研究”と、それを応用して患者さんを対象として行う“臨床研究”は医学研究の２本柱であり、“トランスレーショナルリサーチ”とも呼ばれています。北海道大学病院は平成 24 年に厚労省より臨床研究中核病院に指定され、研究を推進するシステムが整備されています。私たちの診療科でも難治性癌や再発癌を克服するための様々なトランスレーショナルリサーチを展開しており、手術を主軸にし難治癌の集学的治療のエビデンスを創出していきたいと考えています。

3

進行・再発癌に対する新規癌ワクチン CHP-MAGE-A4 の臨床応用と特異的免疫反応の解析

土川　貴裕（北海道大学大学院消化器外科学分野 II）
Tsuchikawa Takahiro
京極　典憲（北海道大学大学院消化器外科学分野 II）
Kyogoku ◎
我孫子剛大（北海道大学大学院消化器外科学分野 II）
Abiko ◎
和田　雅孝（北海道大学大学院消化器外科学分野 II）
Wada Masataka
宮谷内健吾（北海道大学大学院消化器外科学分野 II）
Miyauchi Kengo
池田　裕明（三重大学がんワクチン治療学/遺伝子・免疫細胞治療学講座）
Ikeda Hiroaki
影山　愼一（三重大学がんワクチン治療学/遺伝子・免疫細胞治療学講座）
Kageyama Shinichi
珠玖　　洋（三重大学がんワクチン治療学/遺伝子・免疫細胞治療学講座　教授）
Suku Hiroshi
平野　　聡（北海道大学大学院消化器外科学分野 II　教授）
Hirano Satoshi

— Summary —

I. はじめに

固形がんに対する標準治療法は手術、化学療法、放射線療法であるが進行再発癌に対しては十分な治療成績を示しているとは言えない。このような難治性癌に対する補助療法として免疫治療が有効である可能性があり、当科では 2005 年より難治性再発性の進行癌患者を対象に「HER2 蛋白 CHP 複合体パルス樹状細胞を用いたがんワクチン第 I 相臨床試験」、「NY-ESO-1 等癌抗原蛋白／CpG／リポソーム複合体を用いたがんワクチン第 I 相臨床試験」などの臨床研

究を展開してきた。この中で2009年より進行・再発癌患者を対象にCHP-MCP癌ワクチンの第1+2相臨床研究を行っており、ワクチン接種により誘導される特異的免疫反応について解析している（UMIN000001999）。CHP-MAGE-A4ワクチンは、多様な癌腫に発現が認められている癌精巣抗原MAGE-A4蛋白（表1）をコレステリル基置換プルラン（cholesteryl pullulan:CHP）と結合（図1）、ミセル化した蛋白質複合体で、MAGE-A4蛋白をMHC class Iとclass IIの両経路に効率的に抗原提示させる作用がある（図2、3）[1]。また、これまでの臨床研究の結果から高率に抗原特異的な免疫反応を誘導することが報告されている（表2）[2,3]。

Cancer types	MAGE-A4 expression (%)
Uterine cancer	63
Ovarian cancer	57
Esophageal cancer	52
Head and neck cancer	51
Melanoma	40
Bladder cancer	38-73
Non small cell lung cancer	28-34
Colorectal cancer	22
Hepatic cell carcinoma	20

表1. 各癌腫におけるMAGE-A4抗原の発現頻度

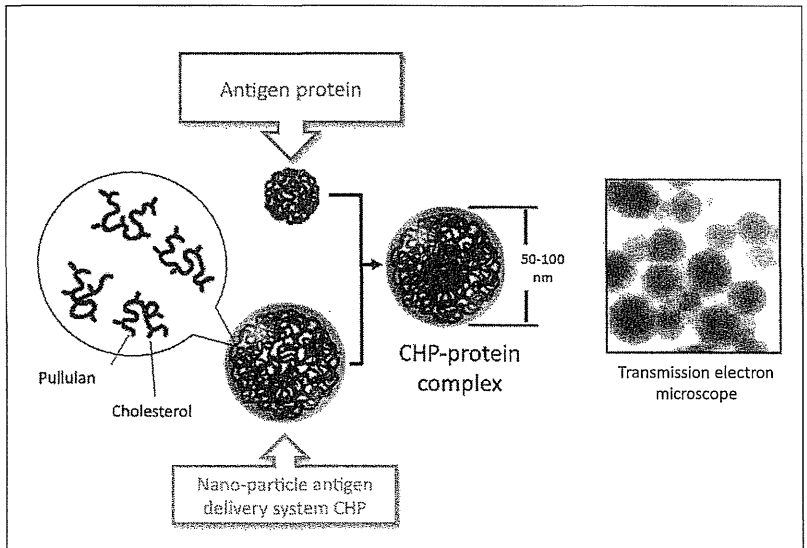


図1.

CHP（cholesteryl pullulan:CHP、コレステリル基置換プルラン）は疎水化多糖類であり、内部に抗原タンパク質を包埋し、ナノ複合体を形成する。

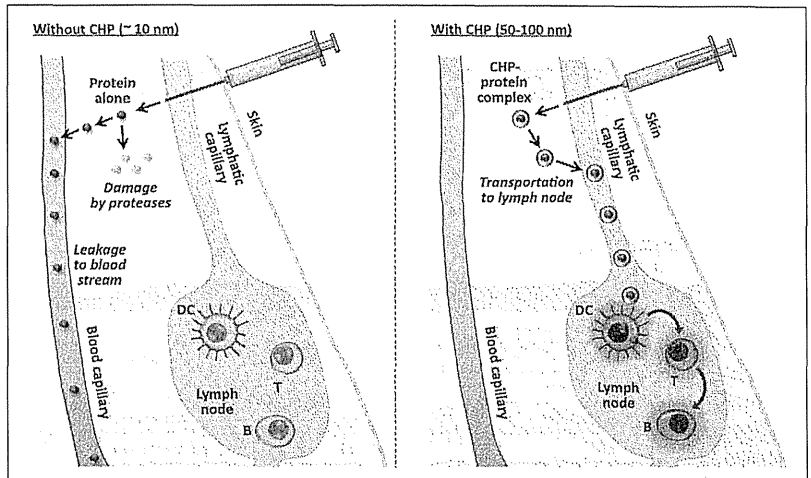


図2.

CHP ワクチンを注射すると、皮膚に存在する樹状細胞などの抗原提示細胞にがん抗原情報が効率よく提示される。

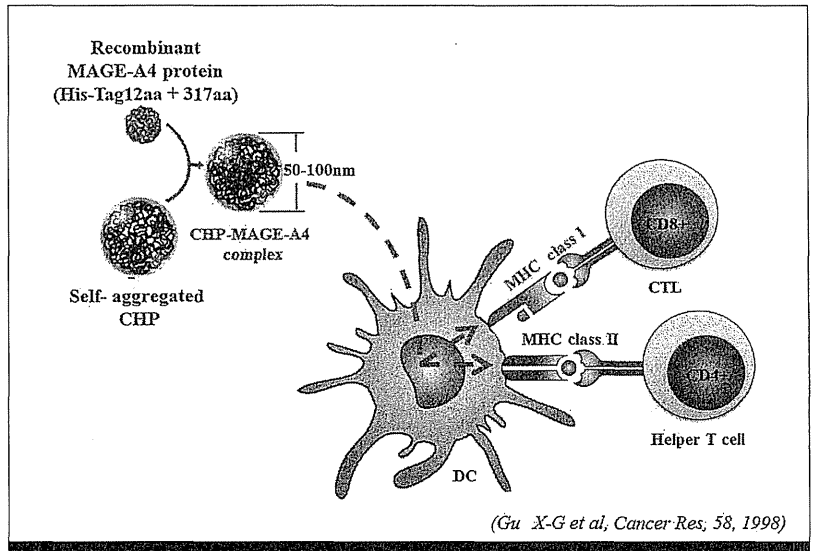


図3. Cholesteryl pullulan(CHP)

CHP-MAGE-A4 ワクチンは癌精巣抗原 MAGE-A4 蛋白を CHP と結合、ミセル化した蛋白質複合体で、MAGE-A4 蛋白を MHC class I と class II の両経路に効率的に抗原提示させる作用がある。従来の抗原タンパクのみの癌ワクチンは、外来抗原であるため class II pathway を介した CD4+Tcell の活性化を担っていた。これに対し、CHP-抗原タンパクの複合体は樹状細胞に取り込まれると、CD4+Tcell はもちろんだが、class I pathway を介し CD8+Tcell も活性化することが可能であり、新規抗原蛋白デリバリーシステムとして期待されている。

Ⅱ. 対象と方法

対象は 2009 年 4 月から 2011 年 11 月までに第 1+2 相臨床研究に登録した進行・再発癌患者 22 例。免疫染色による MAGE-A4 陽性症例を対象とし、2 週間隔で合計 6 回、ワクチンを皮下投与した。主評価項目として安全性、最大耐性量、用量制限毒性の検証、副次的評価項目

MHC (HLA)	Epitope peptide	Position	References
Class I			
A*0101	EVDPASNTY	169-177	Kobayashi, 2003
A*0201	GVYDGREHTV	230-239	Duffour, 1999
A*2402	NYKRCFPVI	143-151	Miyahara, 2005 Ottaviani, 2006
B*3701	SESLKMIF	156-163	Zhang, 2002
B*4002	WEELGVMGV	223-231	Naota, 2006
Class II			
DPB1*0501	AETSYVKVLE HVVRVNARVR	280-299	Ohguri, 2009
DPB1*0901	VRVNARVRIAY	292-302	Naota, not published
DRB1*0403	EHVVRVNARVRIAYP	289-303	Hirayama, not published
DRB1*0803	EHVVRVNARVRIAYP	289-303	Hirayama, not published
DRB1*0901	IFGKASESLKM	151-161	Naota, not published
DRB1*1403	AETSYVKVLE HVVRVNARVR	280-299	Ohguri, 2009

表2. これまでに報告されているMAGE-A4エピトープ

として MAGE-A4 特異的な免疫反応誘導能、臨床効果について検証した。MAGE-A4 の発現評価は MCV-1 抗体を 1 次抗体として用いて免疫染色を施行した。

Ⅲ. 結　果

Ⅲ-1　患者背景

登録患者 22 名の原疾患は大腸癌 13 例、乳癌 3 例、胆道癌 2 例、食道癌、膵癌、乳頭部癌、悪性中皮腫各 1 例であり、年齢中央値は 63 歳(34-79 歳)。全例で切除検体または生検検体での MAGE-A4 抗原発現が確認された。

Ⅲ-2　ワクチン投与による有害事象の評価

第 1 相試験での CHP-MAGE-A4 用量増加投与 (Group1:CHP-MAGE-A4 100ug, Group2: CHP-MAGE-A4 300ug, Group3:CHP-MAGE-A4 300ug+ピシバニール 0.5KE)では、いずれも Grade3(CTCAE ver.3)以上の有害事象を認めなかった。また第 1 相部分のワクチン投与用量増加により早期より特異的免疫反応が誘導される傾向を認めたことから、至適投与用量を CHP-MAGE-A4:300ug+ピシバニール 0.5KE とし第 2 相部分の同一投与量による特異的免疫反応誘導能の評価に移行した。

Ⅲ-3　ワクチン投与症例おける MAGE-A4 抗原特異的免疫反応の解析

臨床研究期間中に 6 回の同一ワクチン投与（CHP-MAGE-A4: 300ug+ピシバニール 0.5KE）を完遂し得た症例 12 例を特異的免疫誘導の解析対象とした。内訳は、男性 10 例、女性 2 例。年齢中央値 63 歳。疾患内訳は大腸癌 7 例、胆道癌 2 例、膵癌 1 例、乳癌 1 例、悪性中皮腫 1 例。RECIST 分類による臨床効果は SD 7 例、PD 5 例であった。12 例中 11 例で MAGE-A4 特異的 I g G サブクラス（IgG, IgG1 , IgG2 , IgG3 , IgG4 ）のいずれかの陽転化が認められ、特異的免疫反応が誘導されたと考えられた。MAGE-A4 特異的 IgG 及び IgG サブ

クラスの抗体価はワクチン接種回数を経るとともに有意に増加したが、患者予後とは相関しなかった。また、MAGE-A3、NY-ESO-1 抗原の発現と抗体反応の推移も同時に検証し、CHP-MAGE-A4 の投与によりこれらの癌抗原に対する抗体反応も誘導されていた。

Ⅳ. がんワクチン投与後の抗原特異的抗体価上昇の意義

T. Boon (4)らにより癌抗原が発見されて以降、担癌宿主内での腫瘍に対する免疫反応の推移や癌ワクチン投与による特異的免疫反応の誘導が研究され、同時に施行されてきた臨床研究の結果と相まってその安全性や有効性についての知見が集積されてきている[5]。我々が施行した CHP-MAGE-A4 に関する研究では第１相部分でその安全性が確認され、また容量増加試験の結果から至適投与量として CHP-MAGE-A4: 300ug+ピシバニール 0.5KE であることが示された。引き続き行われた第２相部分の結果からワクチン投与を受けた生体内では高率に MAGE-A4 特異的な抗体反応が生じていること、さらに MAGE-A3,NY-ESO-1 など他の癌精巣抗原に対する抗体反応も出現することがわかった。このような液性免疫の誘導[3]や antigen spreading(6)は NY-ESO1 や HER2 など他のがんワクチン臨床試験でも報告されており、特異抗原を用いたがんワクチン投与により抗原特異的免疫反応が高率に誘導されると考えられる。しかしながら、このように生体内で腫瘍特異的免疫反応が誘導されるにもかかわらずがんワクチン単独では有意な臨床効果が得られるまでには至っていない。この理由としては HLA-class I down regulation による腫瘍の免疫逃避機構[7]や MDSC、Treg などの抑制性細胞の存在などが知られている。一方で、今回の登録症例の中でも MAGE-A4 特異的抗体価の上昇とともに約５か月間の長期ＳＤが得られた症例も実際に存在し、またがんワクチンが無再発生存期間や全生存期間の延長に寄与するとする報告もあり、今後はこのようながんワクチン反応例を予測するバイオマーカーの検索により適格症例を選択する試み[8]や、より強力な免疫反応を誘導するアジュバントの開発と検証が必要であると考えられる[9]。

おわりに

メラノーマに対して骨髄抑制をきたすような前治療を加えたうえでの養子免疫治療の良好な治療成績や(10)、PD-1 抗体をはじめとする checkpoint block 製剤の認可と臨床導入など[11]、ここ数年で免疫治療は格段に進歩している。がんワクチンとこれらの抗原特異的リンパ球治療や PD-1 製剤と併用することで治療成績の上乗せ効果が期待できるとする報告もあり[12]、これからは製剤自体の工夫はもとより、どんな免疫治療を（がんワクチン、特異的リンパ球など）、どのような適格症例に（バイオマーカーによる患者選択）、いつ導入するか（術前 or 術後補助療法）、そしてどのように投与するのか（化学放射線療法や CTLA-4 阻害薬などと併用して）を組み合わせた集学的な治療が必要であると考える。

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【Doctoral Treatise】

Association of NY-ESO-1 expression with T cell infiltration in the tumor microenvironment of esophageal squamous cell carcinoma and survival

Yoshihiro MURAKAMI
Department of Gastroenterological Surgery II
Hokkaido University Graduate School of Medicine
Sapporo 060-8638, Japan
[Director: Prof. Satoshi Hirano]

Introduction

Esophageal carcinoma remains a disease with poor prognosis. Advances in surgical techniques and perioperative management have improved survival to some extent. The overall 5-year survival rate, however, generally remains less than 50%, even with the use of multimodality therapy [1–3]. Therefore, a novel therapeutic modality is needed, and cancer vaccination is an excellent candidate[4].

A number of human tumor antigens have been identified since the MAGE-A antigen recognized by cytotoxic T lymphocytes (CTLs) was first identified in malignant melanoma [5][6]. Among these antigens, the cancer/testis antigens (CTAs) have received particular attention as potential vaccine targets because of their unique tissue expression. NY-ESO-1 was originally discovered in esophageal carcinoma [7] and is frequently expressed in a variety of cancers but not normal adult tissues except testis [8]. Spontaneous humoral and cellular immune responses against NY-ESO-1 can occur in patients with NY-ESO-1 positive tumors [8]. NY-ESO-1 mRNA and protein expression in esophageal squamous cell carcinoma (ESCC) has been found in 32% of patients, with 13% having NY-ESO-1 antibody production [9]. A correlation between the level of NY-ESO-1 mRNA expression and the degree of immunohistochemistry positivity has been confirmed, and the prognosis of patients with NY-ESO-1 positive tumors is better compared with those with NY-ESO-1 negative tumors [10]. Furthermore, clinical trials of cancer vaccination against NY-ESO-1 have been performed for patients with advanced tumors, and NY-ESO-1 specific immune responses have been confirmed. However, only a few patients have shown objective responses such as tumor regression [11–13], and the trials have so far been disappointing [14][15].

We previously reported that infiltration of tumor-infiltrating $CD4^+$ and $CD8^+$ T lymphocytes (TILs) into tumors caused a favorable prognosis in 122 patients with ESCC [16]. In the present study, we have established standardized diagnostic methods to evaluate NY-ESO-1 expression in ESCC. Immunohistochemical analysis was then performed in the same cohort of 122 surgical specimens reported previously, and correlations with clinical and histopathologic factors were assessed. In particular, we examined the correlation between $CD4^+$ or $CD8^+$ T cell infiltration and NY-ESO-1 expression in the tumor microenvironmenrt of ESCC. Thus, we have obtained beneficial information to develop a strategy for cancer vaccination.

Materials and Methods

1. Cell lines and culture conditions

Human esophageal squamous cell carcinoma cell lines TE2, TE5, TE8, TE10 and TE13 were generously provided by Dr. Nishihira T (University of Tohoku,

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Japan). HEC46 was provided by Dr. Toge T (University of Hiroshima, Japan), and SGF7 was provided by Dr. Saito T (Toyama Medical and Pharmaceutical University, Japan). The human embryonic kidney cell line HEK293 (Clontech Laboratories, Mountain View, CA), TE2, TE5, TE8, TE10, TE13, and HEC46 cells were grown in Dulbecco's modified Eagle's medium (D-MEM, Sigma-Aldrich Co., Ltd., Irvine, CA) with 10% fetal bovine serum (FBS), and 1% penicillin/streptomycin (p/s). SGF7 cells were maintained in RPMI-1640 medium (Sigma-Aldrich Co., Ltd.) with 10% FBS and 1% p/s. All cell lines were maintained in a humidified incubator with 5% CO_2 in air at 37°C.

2. Mice and tumor xenograft models

CB17/SCID mice were obtained from Charles River Japan (Yokohama, Japan). All mice were female and were used at 4–6 weeks of age. Esophageal cell lines (5×10^6) were subcutaneously injected in a volume of 100 μl PBS into the left flank region of each CB17/SCID mouse. When tumor diameter exceeded 10 mm, mice were sacrificed and tumors were separated into 2 blocks; one block was frozen using liquid nitrogen to extract mRNA for RT-PCR, and the other was immersed in formalin for immunohistochemical analysis. TE8 and HEC46 tumors could be established but the other lines could not.

3. Tissue samples

Tumor and normal tissue samples were snap-frozen and stored at –80°C. The samples were obtained in 2002 from resections directly after surgery. Five-micrometer frozen sections were stained with hematoxylin and eosin (HE) to verify ESCC and approximately 100 mm^3 (100 mg) of the tumor block was processed for RNA extraction using the TRIZOL Reagent (GIBCO BRL, Grand Island, NY). As biopsy specimens, 1-mm^3 (1 mg) frozen sections were processed for RNA extraction.

4. Reverse transcriptase polymerase chain reaction (RT-PCR)

Total cellular RNA was isolated with the TRIZOL Reagent (GIBCO BRL) from each cell line. Each 20-μl cDNA synthesis reaction contained 1 μg of total RNA, 1 × First Strand Buffer (GIBCO BRL); 50 mM Tris-HCL, pH 8.3, 75 mM KCL, 3 mM $MgCl_2$), 0.5 mM of each deoxynucleotide triphosphate, 200 units of SUPERSCRIPT II (GIBCO BRL), 10 mM dithiothreitol, and 0.5 μg oligo (dT) (GIBCO BRL). The reverse transcription (RT) reaction was carried out for 50 min at 42°C and inactivated by heating at 70°C for 15 min. Multiplex polymerase chain reaction (PCR) was performed as described previously [17]. Briefly, each 25-μl reaction contained 1 μl of RT reaction products, 1 unit of Taq-Gold DNA polymerase (Boehringer, Mannheim, Germany), 1 × PCR buffer (Boehringer), 160 mM of each deoxynucleotide, and 20 pmol of each 3' and 5' primer specific for NY-ESO-1 (sense, 5'-CAGGGCTGAATGGATGCTGCAGA-3'; antisense 5'-GCGCCTCTGCCCTGAGGGAGG-3'), GAPDH (sense, 5'-ACCCCTTCA TTGACCTCAACT-3'; antisense5'-TGAGTCCTTCCACGATACCAA-3').

NY-ESO-1 and GAPDH cDNA were amplified for 35 cycles. Conditions for NY-ESO-1 PCR were 95°C for 1 min, 62°C for 1 min, then 72°C for 1 min. Conditions for GAPDH PCR were 94°C for 30 sec, 51°C for 30 sec, then 72°C for 30 sec. All PCR products were electrophoresed in a 2.0% agarose gel and visualized by ethidium bromide staining. Human testis cDNA was used as a positive control.

5. Real-time quantitative PCR assay

A reaction mixture containing 250 ng TaqMan probe, 25 μg qPCR Mastermix Plus (Eurogentec, Liege, Belgium), and cDNA in a total reaction volume of 50 μl was prepared. After enzyme activation for 10 min at 95℃ , 50 two-step cycles were performed (20 sec at 95℃ and 60 sec at 64℃) by an ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster, CA). Primers and TaqMan probe were designed as follows: NY-ESO-1-forward, 5'-GGCTGAATGGAT GCTGCAGA-3'; NY-ESO-1-reverse, 5'-CTGGAGACA GGAGCTGATGGA-3'; and TaqMan probe, 5'-FAM-TGTGTCCGGCAACATACTGACTATCCGA-TAMRA-3'. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was measured by GAPDH genomic control (Eurogentic) for normalization. To transform the cycle threshold (Ct) values into absolute mRNA copy numbers, we used a dilution series of linearized plasmid containing the NY-ESO1 insert and constructed a calibration curve. Wells with no template were used for negative control.

6. Patients and esophageal specimens for immunohistochemistry

Surgical specimens from 122 patients who had undergone radical esophagectomy were included in the current study, and findings were referred to the patients' clinical records. To assess the correlation between NY-ESO-1 expression and immune response of the host, the same cohort as reported previously [16] was used. All informed consent processes for this study were conducted in accordance with the guidelines of the Hokkaido University Institutional Review Board.

7. Antibodies

Mouse monoclonal antibody (mAb) against NY-ESO-1 protein (clone E978) was obtained from Invitrogen (Carlsbad, CA). Clone E978 (IgG1) was generated against a 23 kD NY-ESO-1 recombinant protein and specifically detects NY-ESO-1 as described previously [7]. The human CD4-specific mAb [Histofine CD4 mouse immunoglobulin (Ig)G1 mAb], the human CD8-specific mAb (Histofine CD8 mouse IgG1k mAb) were purchased from Nichirei Corporation (Tokyo, Japan) and the human NK cell-specific mAb (Leu 7) were purchased from Becton Dickinson Immunocytometry System (San Jose, CA, USA).

8. Immunohistochemical staining

Immunohistochemical reactions were carried out using the streptavidin-biotin-peroxidase method. The staining methods for CD4, CD8 and NK cell have been described previously [16]. In the case of staining with E978, paraffin-embedded sections were stained with 2.5 μg/ml E978 at a 1:500 dilution in PBS. Each sections were deparaffinized in xylene, washed in PBS (pH 7.4), and rehydrated in a graded series of ethanol solutions. Endogenous peroxidase activity was blocked by a 10-min incubation with 3% hydrogen peroxide in methanol. After washing in PBS, specimens were saturated with 10% normal goat serum (Histofine SAB-PO kit; Nichirei Corp., Tokyo, Japan) for 5 min and then incubated at room temperature for 30 min with primary antibody. After washing in PBS, a biotinylated goat anti-mouse immunoglobulin antibody (Histofine SAB-PO kit; Nichirei Corp.) was applied for 60 min at room temperature. Immunohistochemical reactions were visualized with freshly prepared 3,3-diaminobenzidine tetrahydrochloride (Histofine SAB-PO kit; Nichirei Corp.). Slides were counterstained with hematoxylin and mounted on coverslips. The negative reagent control for NY-ESO-1 consisted of serial tissue sections of each sample, in which isotype-matched negative control mouse IgG1 was used as a primary antibody. A positive or negative tissue control for NY-ESO-1 is described later. The number of stained cells was determined under a microscope (Olympus Optical Co., Ltd., Tokyo, Japan) in three visual fields, at 200 × magnification. When more than 1% of the cytoplasm of all the tumor cell population were stained, the tumor was considered NY-ESO-1 positive. The current study was performed in a retrospective manner, while all specimens were evaluated by three investigators who were blinded to the patients' clinical information.

9. Evaluation and classification of $CD4^+$ T Cells, $CD8^+$ T cells, and NK cells

The immunostained sections were evaluated under a microscope(Olympus Optical Co., Ltd., Tokyo, Japan). Immunochemistry and evaluation of immune cells were done according to the previous reports of Naito *et al.* [18] and Schumacher *et al.* [19], with some modifications. The degree of immune cell infiltration was observed in more than 10 independent high-power (×200) microscopic fields for each tissue sample. The five areas with the most abundant distribution were selected. The number of $CD4^+$ T cells, $CD8^+$ T cells, and NK cells was counted both in the mesenchymal stroma and within the cancer cell nest. In the present study, the threshold values used to demarcate group boundaries were selected such that each group contained equal numbers of patients.

10. Statistical analysis

Either the chi-square test or extended Fisher's exact test were used to analyze the correlation between NY-ESO-1 expression and patients' parameters, including histopathological findings. The Mann-Whitney U test was used for statistical significance between the number of immune cells divided on the basis of NY-ESO-1 expression. The Kaplan-Meier method was used to generate survival curves, and survival differences were analyzed with the log-rank test, based

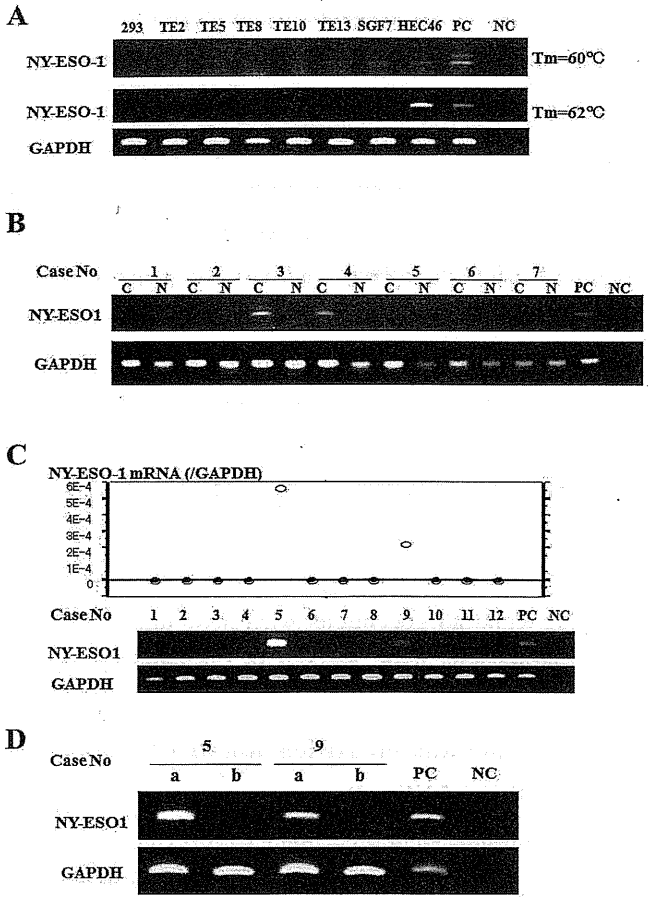

Fig. 1 A) RT-PCR analysis of NY-ESO-1 mRNA expression in human HEK293 cell and ESCC cell lines. Glyceralddehyde-3-phosphate dehydrogenase (GAPDH) was used as the control. Only HEC46 cells express NY-ESO-1 mRNA. **B)** RT-PCR analysis of NY-ESO-1 expression in six different samples of normal esophageal mucosa (N), tumor tissues (C), and positive control are shown. **C)** Real-time-PCR analysis of NY-ESO-1 expression levels in twelve different samples of esophageal tumor tissues are shown. **D)** RT-PCR analysis of NY-ESO-1 expression in two different samples of esophageal tumor tissues, from which 0.1 g of the tumor block (a) or biopsy specimen (b), and positive control are shown.

on the status of NY-ESO-1 expression. Univariate and multivariate analyses of NY-ESO-1 immunoreactivity and clinicopathological features were performed using the Cox proportional hazard regression model. Probability values of less than 0.05 were regarded as indicating significance. These analyses, except the extended Fisher's exact test, were performed using statistical analysis software (Statview J version 5.0; SAS Institute Inc., Cary, NC), and free software from a website (http://aoki2.si.gunma-u.ac.jp/exact/exact.html) was used for the extended Fisher's exact test.

Results

1. Expression of NY-ESO-1 in ESCC cell lines

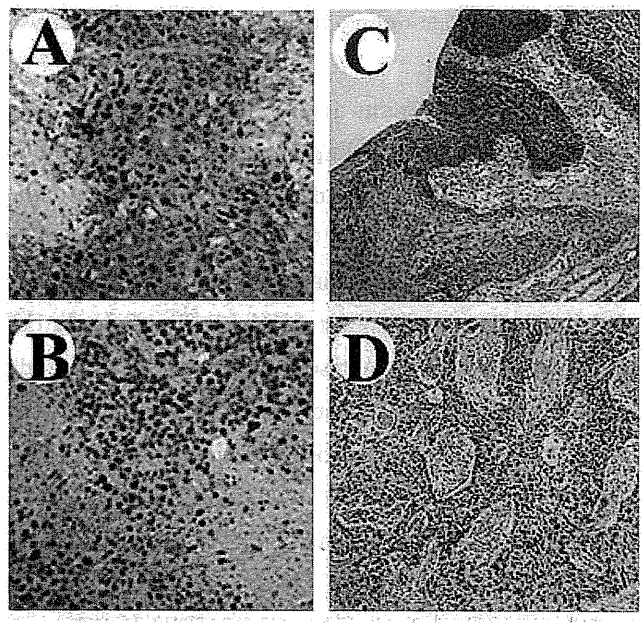

Fig. 2 Immunohistochemical staining for NY-ESO-1 of xenograft tumors and human ESCC samples using E978 antibody. **A)** HEC46 xenograft tumor from SCID mice. **B)** TE8 xenograft tumor. **C)** A case with 5% of cancer cell cytoplasm stained. **D)** No cancer cells stained.

NY-ESO-1 RT-PCR fragments were amplified using mRNA of HEC46 but not of the other 7 cell lines (Fig. 1A). Nonspecific bands were observed under the condition of Tm 60℃, but they disappeared at Tm 62℃. Hereafter, HEC46 cell line was utilized as NY-ESO-1 positive control.

Table 1. Relationship between clinicopathologic features and NY-ESO-1 expression in surgical specimens of ESCC

variables		Number of cases	NY-ESO-1		p-value
			negative (n=100)	positive (n=22)	
Gender	male	105	88	17	0.1891
	female	17	12	5	
Age	≥60	79	60	19	0.0252*
	<60	43	40	3	
p-stage	I	38	30	8	0.9200*
	II	36	29	7	
	III	27	23	4	
	IV	21	18	3	
Grade	1	32	27	5	0.3885*
	2	62	48	14	
	3	28	25	3	
p-T	1	54	41	13	0.3547*
	2	13	10	3	
	3	43	38	5	
	4	12	11	1	
p-N	N0	60	50	10	0.6994
	N1	62	50	12	
p-M	M0	101	82	18	0.6235*
	M1	21	19	3	
TIL	CD4/8(+/+)	44	30	14	0.0029
	Others	78	70	8	

* Extended Fisher's Exact Test

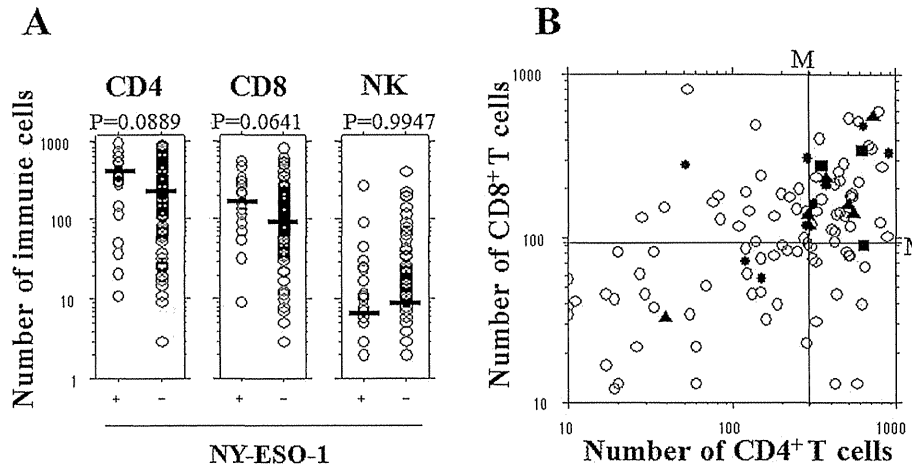

Fig. 3 Correlation between NY-ESO-1 expression and the number of TILs in patients with ESCC. **A**) Comparison of the number of $CD4^+$ T cells, $CD8^+$ T cells and NK cells divided on the basis of NY-ESO-1 expression. The p-values were calculated by Mann-Whitney U test. The bars show the median number of each TIL. **B**) Correlation among the number of $CD4^+/CD8^+$ T cells and NY-ESO-1 expression in patients with ESCC. Open circles: NY-ESO-1 negative patients; filled circles: 0 < NY-ESO-1 positive cells ≤ 10%; filled triangles: 10% < NY-ESO-1 positive cells ≤ 50%; filled squares: 50% < NY-ESO-1 positive cells ≤ 100%. M: median. Higher frequency of NY-ESO-1 positive patients was observed in the CD4/CD8(+/+) group (p=0.0029; chi-square test).

2. RT-PCR of NY-ESO-1 in human tissues

In esophageal sample tissues, NY-ESO-1 RT-PCR fragments were amplified from 10 out of 42 tumor tissues, but not from any normal esophageal mucosa (Fig. 1B).

In the Q-PCR assay, mRNA of NY-ESO-1 was detected from NY-ESO-1 positive cases in the RT-PCR assay, but not from NY-ESO-1 negative cases (Fig. 1C). NY-ESO-1 RT-PCR fragments were not amplified in any biopsy specimens even if the samples were from NY-ESO-1 positive patients (Fig. 1D).

3. NY-ESO-1 expression in xenografts

By the present method, both HEC46 and TE8 tumors obtained from SCID mice showed nuclear staining (Fig, 2A, B). As cytoplasmic staining was observed in HEC46 (Fig. 2A) but not TE8 (Fig. 2B), we defined cytoplasmic staining as a positive control for NY-ESO-1 immunoreactivity.

4. Immunohistochemical analysis in human tissues

We subsequently performed immunohistochemical analysis on the 122 ESCC specimens. NY-ESO-1 immunoreactivity was observed in the cancer cell cytoplasm, as seen in a previous study [10]. Tumor cells of 22 patients (18.0%) were categorized as positively stained for NY-ESO-1 (Fig. 2C) and 100 patients (82.0%) were negatively stained (Fig. 2D). Nuclear staining was observed in all patients (Fig. 2C, D). In about half of patients expressing the NY-ESO-1 antigen, immunohistochemistry stained less than 10% of tumor cells. Dysplasia was found in 45 of 122 specimens; however, NY-ESO-1 expression was not observed in any samples (data not shown).

5. Statistical analyses for NY-ESO-1 expression and clinicopathological data

Correlations between NY-ESO-1 expression and various clinicopathological features are summarized in Table 1. NY-ESO-1 expression was found to correlate with age (p=0.0252). However, no significant correlation was found between NY-ESO-1 expression, gender, pathological data on T classification, N classification, M classification, p-stage grouping, or histopathological grading. The numbers of tumor-infiltrating $CD4^+$ T cells, $CD8^+$ T cells and natural killer (NK) cells divided on the basis of NY-ESO-1 expression were plotted on a scatter plot graph (Fig. 3A). Although no significant correlation was observed between NK cells and NY-ESO-1 expression (p=0.9947), $CD4^+$ T cells (p=0.0889) and $CD8^+$ T cells (p=0.0641) tended to be increased in NY-ESO-1 positive patients. The numbers of $CD4^+$ T cells and $CD8^+$ T cells were plotted, and the distribution from NY-ESO-1 positive patients is shown as filled marks in Fig. 3B. A higher frequency of NY-ESO-1 expression was found in the CD4/8(+/+) group and a significant correlation was observed (p=0.0029) by chi-square test.

A tendency for favorable prognosis was found in

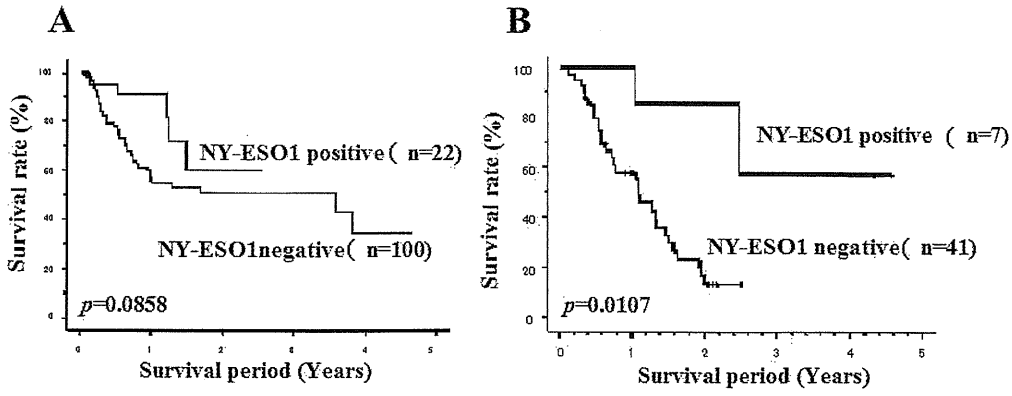

Fig. 4 Kaplan-Meier analysis of survival divided on the basis of NY-ESO-1 expression. **A**) Comparison of overall survival curves for patients with NY-ESO-1 positive and negative tumors out of patients with all stages (n=122). **B**) only stage Ⅲ and Ⅳ ESCC (n=48).

NY-ESO-1 positive patients compared to NY-ESO-1 negative patients (p=0.0858, Fig. 4A). Among stage Ⅲ or Ⅳ disease patients, the overall survival rate was significantly better in patients with NY-ESO-1 positive tumors than in those with NY-ESO-1 negative tumors (p=0.0107, Fig. 4B). Univariate analysis using the Cox proportional hazard model identified NY-ESO-1 expression (p=0.0212) and the status of CD4/8 (p=0.0028) as prognostic predictors. Multivariate analysis indicated that NY-ESO-1 expression (p=0.0058) and CD4/8 status (p=0.0399) were independent prognostic factors in patients with stage Ⅲ or Ⅳ ESCC (Table 2).

Discussion

In planning clinical trials of cancer vaccines against NY-ESO-1 expressing tumors, standardization of diagnostic modality for gene expression is critical for finding suitable patients. We initially confirmed the suitability of RT-PCR as a diagnostic for NY-ESO-1 expression in ESCC cell lines and in samples resected from patients.

Table 2. Univariate and multivariate analysis of NY-ESO-1 and pathologic parameters in patients with stage Ⅲ or Ⅳ ESCC (n=48).

Variables	Hazard ratio	p-value
Univariate		
Sex (male/female)	5.356	0.0933
Age (≥60 y/<60 y)	0.757	0.4572
Grade (2,3/1)	1.004	0.9945
PT (2-4/1)	2.217	0.2779
pN (1/0)	4.711	0.1284
pM (1/0)	0.988	0.9740
Adjuvant therapy (no/yes)	1.957	0.0648
Performance status (1,2/0)	1.536	0.4325
CD4/8 [CD4/8(+/+)/others]	0.196	0.0028
NY-ESO-1 (+/−)	0.178	0.0212
Multivariate		
CD4/8 [CD4/8(+/+)/others]	0.213	0.0058
NY-ESO-1 (+/−)	0.197	0.0399

In the present study, nonspecific bands were observed at the theoretical T_m as reported previously [10], but only specific bands remained when the T_m was raised. In comparing RT-PCR and Q-PCR, we found that the sensitivity of RT-PCR is suitable to detect the expression of NY-ESO-1 when sufficient mRNA is obtained for RT-PCR performed under a condition of higher T_m. We adopted a higher T_m and confirmed that NY-ESO-1 is expressed specifically in tumor cells and that its frequency is consistent with previous data [10].

The detection of NY-ESO-1 mRNA from biopsy samples would be useful for selecting patients with unresectable tumors to enter clinical trials for a cancer vaccine. The present data, however, suggest that this is not the case, since only a small quantity of NY-ESO-1 mRNA, which is less than 0.1% of beta-actin mRNA could be obtained from the samples, meaning that most NY-ESO-1 mRNA from a biopsy specimen would be lost through the process of RNA extraction. Interestingly, we could not always detect NY-ESO-1 expression by RT-PCR using biopsy specimens from a HEC46 xenograft tumor; such tumors seldom have heterogeneity in contrast to the human tumor tissues, so this is best explained by the reason mentioned above. The other reason is apparent from our immunohistochemical data.

We evaluated the legitimacy of the detection of NY-ESO-1 expression and confirmed the consistency of RT-PCR with immunohistochemistry. We found that the E978 antibody works high specificity and that the results of immunohistochemistry reflect those of RT-PCR.

In the HEC46 xenograft tumor, since a necrotic region did not show staining for NY-ESO-1, we could not detect NY-ESO-1 expression by RT-PCR using a biopsy specimen from the necrotic area. However, we could detect it by RT-PCR using mRNA derived from paraffin embedded sections that were confirmed to contain viable tumor cells. Therefore, the extraction of sufficient mRNA from viable and NY-ESO-1 expressing regions would be critical for correct diagnosis. Heterogeneity was always seen in NY-ESO-1 expression, the same as in many other genes in tumors resected from ESCC patients. Several previous studies [10][20] have shown that RT-PCR results do not always accord with those of immunohistochemistry, and authors speculate that heterogeneity causes the inconsistency. In the present study, it was strongly shown that one of the reasons for this contradiction is heterogeneity. Although RT-PCR is a useful and sensitive modality to diagnose NY-ESO-1 expression, the ability of detection depends on several factors such as the quantity of mRNA, the size of the specimen, and the viability of tumors. Biopsy sized specimens could be utilized for detecting NY-ESO-1 expression after overcoming several problems described above and obtaining stable and consistent data. Therefore, we should adopt immunohistochemistry for detecting NY-ESO-1 expression using HEC46 as a tissue positive control while referring to data from RT-PCR. HEC46 xenografts from SCID mice could be used as a positive control for NY-ESO-1 immunohistochemistry and TE8 used as a tissue negative control. Xenografts of cancer cell lines would be better controls because the staining intensity of NY-ESO-1 using testis varied in each patient' s sample, but the intensity using HEC46 is stable.

Finally, we assessed the significance of NY-ESO-1 expression and antitumor immunoresponse of the host by immunohistochemistry in the same cohort as that used in a previous report that clarified the significance of infiltrating CD4 or CD8 positive T lymphocytes [16]. From this, we obtained good information for planning immunotherapy. In this cohort of 122 patients, the frequency of NY-ESO-1 expression was almost the same as in a previous study [10]. Even for NY-ESO-1 positive cases, the ratio of cells expressing NY-ESO-1 was several percent in about half of cases. Interestingly, most NY-ESO-1 positive cases belonged to the CD4/8(+/+) group, which did not depend on the ratio of NY-ESO-1 positive cells. These results suggest that host antitumor immunity might recognize NY-ESO-1 expression even in a few cells, and moreover, it could improve postoperative prognosis of the patients. Besides, we should also take into consideration about the tumor-antigen diversity other than NY-ESO-1 antigen [21]. Moreover, various types of immune cells take part in the host immune balance between immune promoting status and immune regulatory status [22–24].

Our previous study showed that the prognosis of ESCC patients with CD4/8(+/+) status is remarkably better than that of other patients [14]. Therefore, antitumor immunity is obviously an important factor. Moreover, this is critical for advanced ESCC patients because all long-term survivors belonged to CD4/8(+/+) group and no long-term survivors were seen in other groups. It was previously reported that patients with NY-ESO-1 positive tumors survived longer; however, the mechanism was still unclear [10]. Our data support the previous report and demonstrate for the first time that there is a correlation between NY-ESO-1 expression and $CD4^+$ or $CD8^+$ T cell infiltration. In the present study, significant differences between survival curves show that antitumor immunity is more critical especially in advanced cases than in earlier cases. Interestingly, there was no significant difference in tumor progression between the CD4/8(+/+) group and the other groups in this cohort. Therefore, we should pay attention to the fact that antitumor immunity neither always has sufficient effect to suppress tumor growth nor improve patient prognosis if the tumor has already grown significantly[25]. However, this also means that the immune effect improves prognosis most strongly after tumor resection even if the tumor stage is Ⅲ or Ⅳ. These data strongly suggest that immunotherapy against cancer, such as a cancer vaccine, should be applied as an adjuvant therapy to patients who undergo surgical operations and who have few residual tumors.

Conclusions

NY-ESO-1 expression should be detected by using immunohistochemistry with suitable positive or negative controls. In patients with ESCC, NY-ESO-1 might be recognized by the immune system of the host and the best response of antitumor immunity is shown after resection. Therefore, a cancer vaccine applied to ESCC patients as a post-operative adjuvant therapy, if NY-ESO-1 expression is adequately detected using resected tumors, should improve prognosis dramatically.

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