IV. 研究成果の刊行物・別刷

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癌免疫
—癌医療での現在と，そして将来的考察—

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は じ め に

身体にもともと備わっている免疫の力で，病気の治療や予防ができればこんなに素晴らしいことはない．実際，インフルエンザ，ポリオなど多くの感染症では日本や世界の多くの人々がワクチンという形で免疫による恩恵を受けており，小児科感染症領域専門の皆さんが一番よくそのことを周知されていることと思う．

一方，癌はそもそも自分の身体のなかにできた細胞の塊だが，そのような癌を同じ自分の免疫システムで治療や予防ができるのだろうか．たいへん難しい問題であったが，この夢のような医学の実現に向かって今日まで世界中で多くの研究がなされてきた．実際，ヒト癌免疫研究は，免疫応答の存在すら疑われていた長い困難な時代をへて，1990 年代以降，分子レベルで着実な進展をとげ，2000 年代に入り，癌患者で臨床試験，治験も盛んに行われ，癌免疫治療・予防が日常臨床で具現化するところまできているといえる．

特に，抗 CD20 抗体に始まった抗体治療が癌治療のあらたな標準として大きく貢献し，ここ 1〜2 年はさらに，T 細胞の免疫制御性分子 CTLA-4 や PD-1 に対する抗体治療が 50〜80％の腫瘍退縮など驚くべき効果を上げつつある．現在，これらは大変なトピックスになっていて，2013 年末に Science 誌は，同年の科学的進展の 10 大ニュースのトップに癌免疫の進展を選んでいる．新聞紙上でも大きく報道された．

I．癌特異的免疫応答

免疫による治療の究極の哲学は，癌に対する特異性である．上記の CTLA-4 や PD-1 などの免疫制御性分子の抗体治療は素晴らしい成果を上げつつあるが，癌特異性という観点からは厳密ではなく，副作用の心配が必ずしも解決されているとはいえない．

癌特異性は理論的には，癌細胞や癌組織にのみ発現する蛋白分子であり，これが基本と考えられる．これらは細胞内でプロテアソームなどにより分解され，ペプチド断片になり，そして HLA A24 とか A2 などの HLA クラス I 分子と細胞内で会合，HLA クラス I 分子-ペプチド断片複合体となり，ゴルジ装置を経由して細胞表面に発現する．これら複合体が cytotoxic T lymphocyte（CTL：細胞障害性 T リンパ球）により認識され，癌細胞の特異的排除につながるわけである．すなわち，癌特異的な遺伝子発現とその蛋白，ペプチド断片が免疫による癌特異応答を規定するわけである．癌ペプチドはこのように癌特異性という意味では最も優れている．

今日的には癌治療は化学療法がまずは先行しているが，将来的には癌患者が免疫治療を化学療法などで免疫系の抑制がきたされる前に，最初の癌の治療法として選択する可能性も出ると考えられる．

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II．癌特異的ペプチドワクチン

われわれは一貫してヒト癌抗原を研究してきたが，これまでの研究で発見した癌抗原の一つがアポトーシス抑制分子でサバイビン 2B という物質である．この分子は機能的には代表的な inhibitor of apoptosis proteins であり，癌の分裂，増殖を維持させている．われわれはまずこの分子が多くの癌腫で発現し，正常組織で発現がなく，癌特異的であることを確認した．さらに多くの癌患者 T 細胞が本分子由来ペプチドを認識し，優れた癌特異的抗原として癌の免疫学的標的になることを明らかにしてきた．

これらの基礎的成果を基に約 10 年前から大腸癌，膵臓癌，乳癌，肺癌，膀胱癌，口腔癌など，さまざまな癌腫を対象としてこの癌特異的抗原ペプチドの癌ワクチンとしての副作用や，有効性の臨床成績，あるいはワクチン投与による免疫反応の詳細につき臨床教室と共同で行ってきた．その結果，臨床的にも免疫学的にも一定程度の有効性は認められ，大きい希望を抱かせてくれている．この研究も平成 23 年からは第 I 相の臨床試験が厚生労働省の支援の下開始され，さらに平成 25 年秋からは第 II 相の臨床試験が進行膵臓癌を対象に全国規模で行われている．

III．癌幹細胞（cancer stem cells：CSC）/癌起始細胞（cancer-initiating cells：CIC）の存在と免疫標的研究

それでも癌は癌であり，その効率的な治療は当然困難が予想される．一つの癌の抗原ワクチンで癌の大きな制御は難しいことも予想される．

一方，多くの研究により基本的な課題も浮き彫りになってきている．これらの主たるものは，① 癌抗原であろうとウイルス抗原であろうと，抗原特異的リンパ球は体内で無限には増えない．これはゆるぎない生体のホメオスタシスでもあり，このことを理解して，よりすぐれたワクチンを開発することが重要と考えられる．すなわち，患者体内での限りあるリンパ球が標的とする，適切な癌抗原の選択をすることが大変重要と考えられる．

このことに最も合目的な抗原は，その存在が実証されてきている，いわゆる CSC/CIC に発現する癌抗原と考えることができる．CSC/CIC の研究はここ数年飛躍的に進んできた．われわれはすでに 20 年前からこの研究にとりくんできた．

その結果，根元細胞としての特徴を有する CSC/CIC は，T リンパ球の免疫学的なターゲットとして極めて魅力的であることがわかった．特に CSC/CIC 表面の HLA 分子上に提示される特異的癌抗原ペプチドが細胞障害性 T 細胞（CTL）のターゲットになり得るか否か，に大きな焦点があった．

われわれはまず，Hoechst33342 の排泄を指標とした side population（SP）法，ALDH 法，sphere formation 法などにより，CSC/CIC の分離同定をさまざまな癌で行った．続いて CSC/CIC と非 CSC/CIC を比較することにより，CSC/CIC に特異的，選択的に発現する興味ある分子を次々と同定した．しかも正常の幹細胞に発現をみない分子として，以下の興味深い分子を発見した．

それらは，いわゆる癌精巣抗原〔cancer-testis（CT）antigens〕としての特徴を有する① Or7c1（Olfactory receptor family 7 subfamily c member 1），② DNAJB8（DnaJ homolog subfamily, member 8），③ SMCP（sperm mitochondria-associated cysteine-rich protein）などであった．

IV．CSC/CIC と精巣特異抗原

CSC/CIC に精巣特異抗原のいくつかが選択的，特異的に発現する事実は，CSC/CIC の生物学的特徴を考えるうえで大変興味深いものといえる．少なくとも spermatogenesis のある特定の分子機構が CSC/CIC の特徴と明らかにリンクしていることを示唆している．その意味，意義はまだわからないが，癌を考えるうえでとても重要な課題となってきている．

これらの分子は，正常では睾丸組織に発現するのみであり，他方多くの癌細胞で高い発現をみる分子である．しかも CSC/CIC のステムネス特質と直接関連する．これらの遺伝子を高発現させると NOD SCID マウスで高い *in vivo* 造腫瘍性を示す．一方，siRNA 処理によりそれらの特徴を大きく失う．すなわち，CSC/CIC のステムネスの特質

に直接関与する分子であると考えられる.

　これらの分子は癌ワクチンとして活性も鮮やかに示す. 特に, Or7c1 と DNAJB8 分子は CTL の強い誘導性を示し, 腫瘍の免疫学的抑制を *in vivo* でもたらす.

　ここで正常精巣への副作用が気になる. しかし, 癌精巣特異抗原は癌と男性正常精巣に発現しているわけだが, 正常精巣は HLA 分子発現がエピジェネティカルに制御されており, 通常, 発現がない. そのため, CTL は正常精巣を攻撃しないと考えられており, この意味でも理想的な癌ワクチンと考えられる.

　この成果を受けて, 前者 Or7c1 の抗原ペプチドは臨床試験を始めている. これは世界で最初のヒト癌幹細胞/癌起始細胞 (CSC/CIC) ワクチンであり, その臨床的, 免疫学的成果が大変注目されている.

お わ り に

　われわれが探求しているヒト癌幹細胞/癌起始細胞 (CSC/CIC) の特異抗原, すなわちある特定の癌精巣抗原だが, これらは恐らく最も根元的あるいは原始的な腫瘍抗原として生体の免疫監視機構に抗癌制御として機能していると推測している. なぜなら多くの臨床的腫瘍は単発癌で推移する. これら癌精巣抗原が根元的あるいは原始的腫瘍抗原として機能していればこその, このような疫学的事実に通じるものと考えられる.

　また, これは全くの筆者の個人的考えだが, 疫学的に先進国のほとんどで男性の癌死が女性のそれに比し優っている. 例えば, 日本では人口 10 万人比では男性が癌死 300 人前後, 女性が 200 人前後/年である. イタリア, フランスなどもそのような傾向がある. この差は謎だが, 男性がより激しい環境因子に曝されているというような社会的, 職業的側面だけでは説明できないと筆者は推測している. もしかして, 女性の正常組織には全く存在しない, 癌精巣抗原が根元的あるいは原始的腫瘍抗原として女性で癌免疫監視機構に機能しているのでは, と思うのである. Wild だが, しかし大変魅力ある作業仮説と考える.

　いずれにしても癌の免疫治療と予防は次世代に

は具現化すると筆者は予測している. その根拠をこの総説で皆様に理解していただくことができれば大変幸いと思う.

文　献

（2013 以降の論文のみを下記に紹介する. それ以前の詳細は, PubMed で Sato N and Sapporo Medical のキーワード挿入で論文検索できる）

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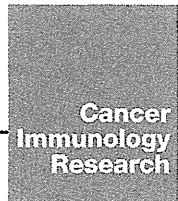
Prognostic Impact of Human Leukocyte Antigen Class I Expression and Association of Platinum Resistance with Immunologic Profiles in Epithelial Ovarian Cancer

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Abstract

Epithelial ovarian cancer (EOC) is one of the most deadly carcinomas in females. Immune systems can recognize EOCs; however, a defect of human leukocyte antigen (HLA) class I expression is known to be a major mechanism for escape from immune systems, resulting in poor prognosis. The purpose of this study is to identify novel correlations between immunologic responses and other clinical factors. We investigated the expression of immunologic components in 122 cases of EOCs for which surgical operations were performed between 2001 and 2011. We immunohistochemically stained EOC specimens using an anti-pan HLA class I monoclonal antibody (EMR8-5) and anti-CD3, -CD4, and -CD8 antibodies, and we analyzed correlations between immunologic parameters and clinical factors. In multivariate analysis that used the Cox proportional hazards model, independent prognostic factors for overall survival in advanced EOCs included low expression level of HLA class I [risk ratio (RR), 1.97; 95% confidence interval (CI), 1.01–3.83; $P = 0.046$] and loss of intraepithelial cytotoxic T lymphocyte (CTL) infiltration (RR, 2.11; 95% CI, 1.06–4.20; $P = 0.033$). Interestingly, almost all platinum-resistant cases showed a significantly low rate of intraepithelial CTL infiltration in the χ^2 test (positive vs. negative: 9.0% vs. 97.7%; $P < 0.001$). Results from a logistic regression model revealed that low CTL infiltration rate was an independent factor of platinum resistance in multivariate analysis (OR, 3.77; 95% CI, 1.08–13.12; $P = 0.037$). Platinum-resistant EOCs show poor immunologic responses. The immune escape system of EOCs may be one of the mechanisms of platinum resistance. Cancer Immunol Res; 2(12); 1220–9. ©2014 AACR.

Introduction

Epithelial ovarian cancer (EOC) is the sixth most common female cancer worldwide and is the leading cause of death in gynecologic malignancies. EOC was comparatively uncommon in Japan, but the number of patients with EOC has been increasing, according to the rise of average body mass index (BMI; ref. 1). EOC is difficult to diagnose and most cases are found in an advanced stage often with peritoneal dissemination or distant metastasis. Prognosis has been improved by platinum-based chemotherapy, which is used as a first-line

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Note: Supplementary data for this article are available at Cancer Immunology Research Online (http://cancerimmunolres.aacrjournals.org/).

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chemotherapy (2); however, patients who are resistant to platinum agents have a very poor prognosis with a median survival period of only 6 months (3). Several mechanisms have been reported to be involved in platinum resistance, including intracellular drug accumulation and/or increased drug efflux, drug inactivation by increased levels of cellular thiols, alterations in drug target, processing of drug-induced damage by increased nucleotide excision-repair activity, and decreased mismatch-repair activity and evasion apoptosis (4–6). Despite the revelation of mechanisms, it is still not clear how platinum resistance in EOC can be overcome.

An interesting study revealed that intratumoral infiltration of CD3^{+} T cells was a prognostic factor for patients with EOC (7). Results from that study suggested that immunologic reaction for EOC has a significant impact on disease control, and cancer immunotherapy has emerged as another treatment modality for EOCs. CD8^{+} cytotoxic T lymphocytes (CTL) recognize an antigenic peptide presented by human leukocyte antigen (HLA) class I. Various immunotherapy trials for EOC have been conducted and reviewed by Sabbatini and Odunsi (8). Prognosis of patients with clear-cell adenocarcinomas was improved by EOC immunotherapy using grypcan-3–derived peptide (9). Furthermore, a phase II trial of anti–PD-1 antibody therapy is ongoing since 2011 (10, 11). However, there are several mechanisms by which cancer cells escape detection

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and destruction by the immune system (12–14). HLA class I loss, a condition in which CTLs cannot recognize cancer cells, is a major mechanism of immune escape. Downregulation or loss of HLA class I molecules in EOC has been reported (15–18), and the mechanism of immune escape is a critical factor in immunotherapy.

Recently, we have generated a monoclonal antibody (mAb) against HLA class I molecules (clone: EMR8-5; ref. 19). EMR8-5 is reactive for HLA-A, HLA-B, and HLA-Cw, whereas clone HC10, a commonly used HLA class I antibody, is reactive for only HLA-B and HLA-Cw (19). Therefore, EMR8-5 is more suitable for evaluation of HLA class I expression. In this study, we examined the expression profiles of HLA class I molecules immunohistochemically using clone EMR8-5 in representative subtypes of EOC, and we analyzed their correlations with infiltration of intratumoral $CD3^+$, $CD4^+$, and $CD8^+$ T cells and various clinical characteristics.

Materials and Methods

Patients and specimens

Surgical specimens were obtained from 122 patients with primary EOC treated at Sapporo Medical University Hospital (Sapporo, Japan) during the period from 2001 to 2011. Written informed consent was obtained from each patient according to the guidelines of the Declaration of Helsinki. Patients underwent abdominal hysterectomy, bilateral salpingo-oophorectomy, omentum resection, lymphadenectomy, and resection of metastatic lesion when possible. All H&E-stained slides were reviewed by a pathologist, and the diagnosis was confirmed in accordance with FIGO (International Federation of Gynecology and Obstetrics) stage. Early cases were defined as stages I and II, and advanced cases were defined as stages III and IV. In advanced cases, platinum-based combination agents were administered as adjuvant chemotherapy. Platinum-resistant cases were defined as those with disease progression during treatment with first-line chemotherapy or relapse within 6 months after completion of chemotherapy. Optimally resected cases were defined as cases with complete tumor resection or with residual tumor of less than 1 cm in diameter. Overall survival was documented for all patients, and survival was calculated from the day of the operation until November 31, 2013.

Immunohistochemical staining

Sections (5 μm in thickness) of formalin-fixed paraffin-embedded tumors were immunostained using mAbs after epitope retrieval by Novocastra epitope retrieval solution pH9. mAb EMR8-5 was used to stain HLA class I molecules (19). For staining of T lymphocytes, we used anti-CD3 (Nichirei; no. 413591), anti-CD4 (Nichirei; no. 413951), anti-CD8 (Dako; no. N1591), and anti-FOXP3 (Abcam; clone SP97) mAbs. EMR8-5 was diluted 1×2,000, anti-FOXP3 antibody was diluted 1×100, and anti-CD3, -CD4, and -CD8 antibodies were already established in working dilution. Subsequent incubation with a secondary biotinylated antibody was performed, and endogenous peroxidase activity was blocked by immersion in 3% peroxidase. Slides were then counterstained with hematoxylin,

rinsed, dehydrated through graded alcohols into nonaqueous solution, and coverslipped with mounting medium.

Quantification of HLA class I staining

Membrane immune reactivity levels for HLA class I were categorized as 0, +1, +2, and +3 in accordance with criteria determined by the HLA expression evaluating consortium (Fig. 1A). A score of zero was defined as <10% membrane staining. A score of +1 was defined as 10% to 50% membrane staining or 10% to 90% of the membrane stained weakly. A score of +2 was 50% to 90% membrane staining or >90% of the membrane stained weakly, and a score of +3 was defined as membrane staining in >90% of the tumor cells. Finally, the quantified HLA class I levels were divided into two groups: scores 0 and +1 as HLA class I low group, and scores +2 and +3 as HLA class I high group.

Quantification of intraepithelial T cells

We counted intraepithelial infiltrated $CD3^+$, $CD4^+$, and $CD8^+$ T cells in high-power fields (HPF; ×400) and calculated their averages. On the basis of the average counts of infiltrated lymphocytes, we classified them into two groups: T-cell infiltration–positive group with ≥ 10 counts/HPF and T-cell infiltration–negative group with <10 counts/HPF (Fig. 1B).

Statistical analysis

Statistical analyses were performed with SPSS (version 11 for Windows; SPSS Inc.), and GraphPad Prism (version 4.0 for Windows; GraphPad Software Inc.) was used for plotting Kaplan–Meier curves. Pearson χ^2 tests were used to determine the significance of associations between characteristic variables. The Spearman rank correlation coefficient was used to evaluate correlations between HLA class I expression and T-cell infiltration. Survival rates were calculated using the Kaplan–Meier method, and differences between groups were tested using the log-rank test. The Cox proportional hazards model was used for multivariate analysis to determine risk ratio and independent significance of individual factors for prognosis. A logistic regression model was used for multivariate analysis to predict odds ratio of individual factors for platinum resistance. Each multivariate analysis was performed with the stepwise method. In all analyses, P values of <0.05 were considered as statistically significant.

Results

Differences in immunologic parameters

The clinicopathologic characteristics of the patients are summarized in Table 1. There were significant differences in HLA class I expression and intraepithelial T-cell infiltration among the histologic subtypes. The clear-cell adenocarcinoma histologic subtype is significantly correlated with lower expression level of HLA class I molecules (HLA class I low; $P = 0.007$) and lower infiltration rates of $CD3^+$ T cells ($CD3^+$ T-cell negative; $P = 0.002$) and $CD8^+$ T cells ($CD8^+$ T-cell negative; $P < 0.001$). The serous adenocarcinoma and endometrioid adenocarcinoma histologic subtypes showed significant correlations with higher infiltration rate of T cells. Interestingly,

Table 1. Clinicopathologic characteristics of cases

Characteristics	HLA class I			CD3+ T cells			CD4+ T cells			CD8+ T cells			Total
	High (n = 79)	Low (n = 43)	P	Positive (n = 52)	Negative (n = 70)	P	Positive (n = 23)	Negative (n = 99)	P	Positive (n = 78)	Negative (n = 44)	P	(N = 122)
Age, y													
Mean ± SD	54.7 ± 10.6	55.6 ± 11.4		55.1 ± 10.63	55.1 ± 9.94		55.1 ± 10.3	55.1 ± 10.7		55.1 ± 11.5	55.1 ± 8.94		55.1 ± 10.2
Range	29–80	30–81		29–81	30–81		29–72	30–81		29–81	33–81		29–81
Parity, %													
0	24 (30.4)	15 (34.9)	0.77	14 (26.9)	25 (35.7)	0.044[a]	11 (47.8)	28 (28.3)	0.043[a]	26 (33.3)	13 (29.5)	0.044[a]	39 (32)
1	17 (21.5)	9 (20.9)		10 (19.2)	16 (22.9)		3 (13.0)	23 (23.2)		13 (16.6)	13 (29.5)		26 (21.3)
2	25 (31.6)	15 (34.9)		15 (28.8)	25 (35.7)		3 (13.0)	37 (37.4)[a]		23 (29.5)	17 (38.6)		40 (32.8)
3	11 (13.9)	4 (9.3)		11 (21.2)[a]	4 (5.7)		5 (21.7)	10 (10.1)		14 (17.9)[a]	1 (2.3)		15 (12.3)
4	2 (2.53)	0 (0)		2 (3.8)	0 (0)		1 (4.3)	1 (1.0)		2 (2.53)	0 (0)		2 (1.64)
Histologic subtype, %													
Serous	40 (50.6)	21 (48.8)	0.85	29 (55.8)	32 (45.7)	0.27	9 (39.1)	52 (52.5)	0.25	45 (57.7)	16 (36.4)	0.024[a]	61 (50)
Clear cell	20 (25.3)	17 (39.5)[b]	0.007[b]	8 (15.4)	29 (41.4)[b]	0.002[b]	4 (17.4)	34 (33.3)	0.11	14 (17.9)	23 (52.3)[b]	<0.001[b]	37 (30.3)
Endometrioid	14 (17.7)	4 (9.3)	0.84	13 (25.0)[b]	5 (7.1)	0.006[b]	9 (39.1)[b]	9 (9.1)	<0.001[b]	15 (19.2)	3 (6.8)	0.06	18 (14.8)
Mucinous	5 (6.33)	1 (2.32)	0.33	2 (3.8)	4 (5.7)	0.64	1 (4.3)	5 (5.1)	0.89	3 (3.8)	3 (6.8)	0.47	6 (4.92)
FIGO stage, n (%)													
I	30 (38)	12 (27.9)	0.53	16 (30.8)	26 (37.1)	0.76	10 (43.5)	32 (32.3)	0.46	23 (29.5)	19 (43.2)	0.31	42 (34.4)
II	3 (3.8)	3 (6.98)		2 (3.8)	4 (5.7)		1 (4.3)	5 (5.1)		3 (3.8)	3 (6.8)		6 (4.92)
III	42 (53.2)	24 (55.8)		31 (59.6)	35 (50.0)		12 (52.2)	54 (54.5)		47 (60.3)	19 (43.2)		66 (54.1)
IV	4 (5.06)	4 (9.3)		3 (5.8)	5 (7.1)		0 (0)	8 (8.1)		5 (6.4)	3 (6.8)		8 (6.56)
Peritoneal dissemination, %	46 (58.2)	25 (58.1)	0.99	35 (67.3)	36 (51.4)	0.08	13 (56.5)	58 (58.6)	0.86	51 (65.4)[b]	20 (45.5)	0.032[a]	71 (58.2)
Lymph node metastasis, %	21 (26.6)	15 (34.9)	0.34	16 (30.8)	20 (28.6)	0.79	6 (26.1)	30 (30.3)	0.69	24 (30.8)	12 (27.3)	0.10	36 (30)
Optimal debulking surgery, %	49 (62.0)	23 (53.5)	0.36	26 (50.0)	46 (65.7)	0.08	18 (78.3)[a]	54 (54.5)	0.037[a]	43 (55.1)	29 (65.9)	0.24	72 (59)
Platinum resistance, %	28 (35.4)	22 (51.2)	0.09	21 (40.4)	29 (41.4)	0.91	5 (21.7)	45 (45.5)[a]	0.013[a]	7 (9.0)	43 (97.7)[b]	<0.001[b]	50 (41)

NOTE: Patients' characteristics were analyzed statistically by the χ^2 test.
Asterisks are pointed on significant larger numbers.
[a]$P < 0.05$.
[b]$P < 0.01$.

Figure 1. Expression of HLA class I molecules and intraepithelial infiltration of T cells. A, HLA class I immunohistochemical staining of EOC cases. Score 0, <10% cancer membrane staining. Score +1, 10% to 50% membrane staining or 10% to 90% of the membrane stained weakly. Score +2, 50% to 90% membrane staining or >90% of the membrane stained weakly. Score +3, membrane staining in >90% of the tumor cells. B, immunohistochemical staining of T cells. T cells were immunohistochemically stained by anti-CD3, -CD4, and -CD8 antibodies. T cells were counted in a HPF (×400). T-cell infiltration–positive group for 10 counts/HPF and T-cell infiltration–negative group for <10 counts/HPF. C, scatter diagrams of HLA class I expression and T-cell infiltration. y-axis is the average of the numbers of intraepithelial T cells in HPFs.
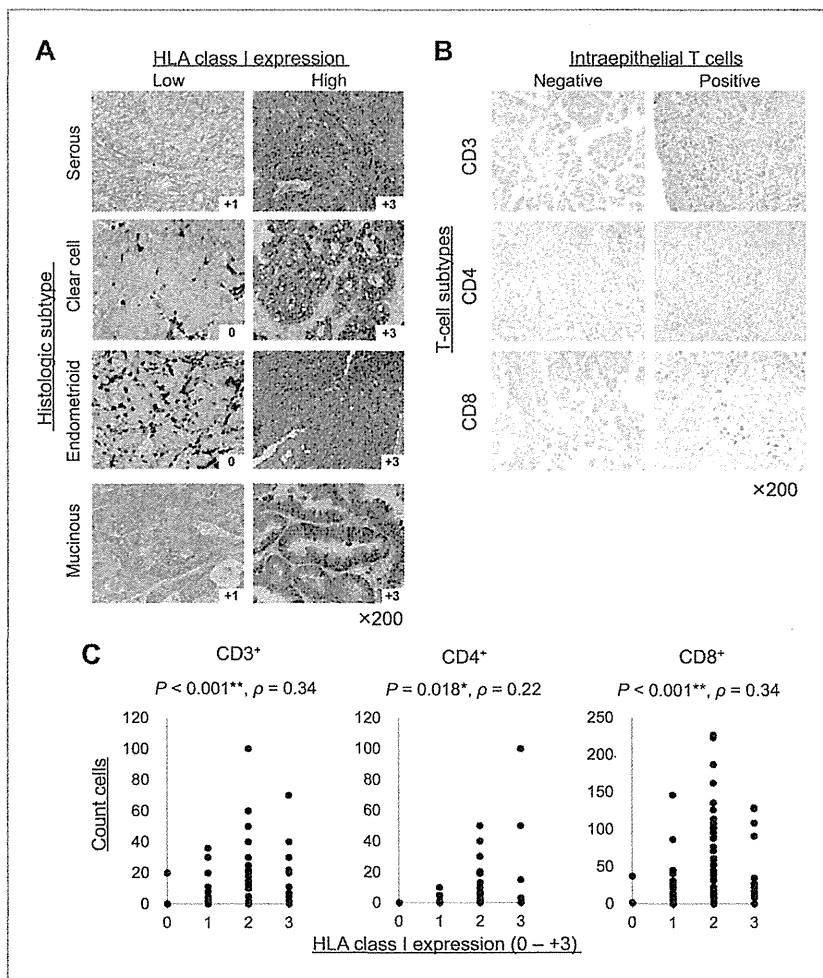
most CD8$^+$ T cell–negative cases showed a significant correlation with platinum resistance (97.7%; $P < 0.001$). As shown in Fig. 1C, HLA class I expression and intratumoral T-cell infiltration showed a positive correlation, but it was very weak. CD3$^+$ and CD8$^+$ T-cell infiltrations showed a stronger correlation than did CD4$^+$ T-cell infiltration ($\rho = 0.34$, 0.34; CD3$^+$, 0.34; CD8$^+$ vs. 0.22; CD4$^+$).

Factors correlated with poor survival rates

Log-rank analyses were performed according to FIGO stages and histologic subtypes (Fig. 2). These analyses revealed that low HLA class I expression level was correlated with poorer prognosis in total cases ($P = 0.004$), advanced cases ($P = 0.032$), and early cases ($P = 0.035$). Low HLA class I was correlated with poorer prognosis in serous adenocarcinoma cases ($P = 0.045$), endometrioid adenocarcinoma cases ($P = 0.039$), and mucinous adenocarcinoma cases ($P = 0.025$), but not in clear-cell adenocarcinoma cases ($P = 0.41$). CD3$^+$ T-cell positivity was

not correlated with prognosis in total cases, advanced cases, early cases, and also in all histologic subtype cases. CD4$^+$ T-cell positivity was correlated with better prognosis in total cases ($P = 0.033$), but not in advanced cases ($P = 0.094$) or early cases ($P = 0.252$). CD4$^+$ T-cell positivity was not correlated with any histologic subtypes (serous, $P = 0.799$; clear cell, $P = 0.206$; endometrioid, $P = 0.065$; mucinous, $P = 0.655$). Because all CD4$^+$ T cell–positive cases with clear-cell, endometrioid, and mucinous subtypes were alive during that time, we reanalyzed the prognosis by nonserous (clear-cell, endometrioid, and mucinous) subcategory, and found that CD4$^+$ T-cell positivity was correlated with better prognosis in nonserous histologic subtype ($P = 0.017$), but not in serous subtype ($P = 0.799$; Fig. 3A). CD8$^+$ T-cell positivity was not correlated with better prognosis in total cases ($P = 0.077$) or early cases ($P = 0.122$), but it was correlated with better prognosis in advanced cases ($P = 0.004$). CD8$^+$ T-cell positivity was not correlated with better prognosis all in histologic subtypes; however, it was
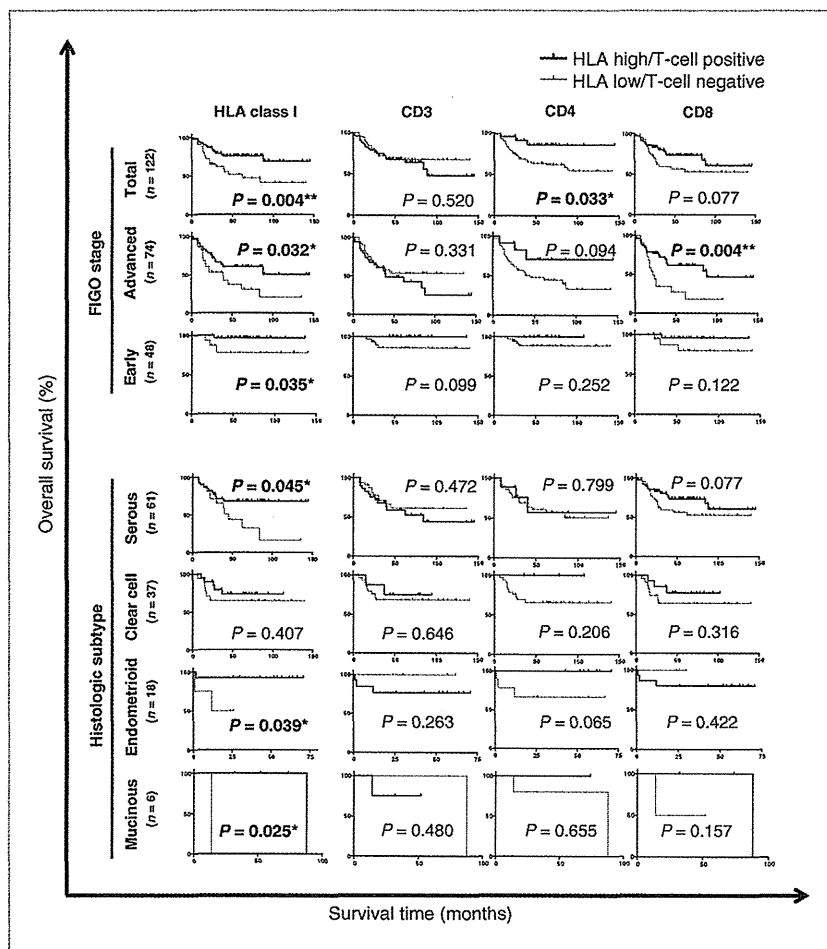
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Figure 2. Kaplan–Meier curves of overall survival rates based on the expression of HLA class I and T-cell infiltration. The cases were analyzed by the expression of HLA, infiltration of CD3^{+} T cells, CD4^{+} T cells, and CD8^{+} T cells. Bold, statistically significant differences. Advanced, stage III/IV; early, stage I/II; serous, serous adenocarcinoma; clear cell, clear-cell adenocarcinoma; endometrioid, endometrioid adenocarcinoma; mucinous, mucinous adenocarcinoma.

correlated with better prognosis in advanced clear-cell adenocarcinoma cases ($P = 0.009$; Fig. 3B). A previous study had suggested that the presence of regulatory T cells (Treg) was correlated with poorer prognosis in ovarian cancer cases (20); thus, we further investigated the CD4^{+} T cell–positive cases using anti-FOXP3 antibody (Supplementary Fig. S1). High Treg infiltration (Treg high) was not correlated with the prognoses ($P = 0.051$); however, high CD8^{+} T cell:Treg ratio showed tendency to be associated with better prognosis ($P = 0.089$). Interestingly, patients with high CD8^{+} T cells:Treg ratios were alive.

To assess whether the immunologic status was an independent marker of prognosis, the relative influence of its expression and other clinical characteristic variables were analyzed by multivariate analysis using the Cox proportional hazard model (Table 2). The cases were divided into three groups as shown in Table 2: total cases, early cases (FIGO stage I/II), and advanced cases (FIGO stage III/IV). HLA class I low was a significant prognostic factor [risk ratio (RR), 2.18; 95%

confidence interval (CI), 1.16–4.12; $P = 0.016$] in total cases. In early-stage cases, factor of final model was restricted only by HLA class I expression, and it did not reach statistical significance in the univariate Cox proportional hazard model (RR, 7.86; 95% CI, 0.82–76.13; $P = 0.074$). However, using log-rank analysis, the early-stage cases also reached statistically significant difference ($P = 0.035$). HLA class I low (RR, 1.97; 95% CI, 1.01–3.83; $P = 0.046$) and CD8^{+} T-cell positivity (RR, 2.11; 95% CI, 1.06–4.20; $P = 0.033$) were significant prognosis factors in advanced cases. Clear-cell adenocarcinoma histologic subtype was an independent prognostic factor in advanced cases (RR, 2.38; 95% CI, 1.03–5.48; $P = 0.042$).

Factors correlating with platinum resistance

A multivariate logistic regression model was used for further analysis. Cases used for the analysis were restricted to advanced cases with adjuvant platinum-based chemotherapy. Those cases that achieved optimal debulking were excluded. As shown in Table 3, CD8^{+} T-cell positivity was correlated with

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Figure 3. Kaplan–Meier curves of overall survival rates based on T-cell infiltration. A, Kaplan–Meier curves for overall survival of total cases. The cases were analyzed by infiltration of CD4[1] T cells. Total cases, $N = 122$; serous adenocarcinoma cases, $n = 61$; nonserous adenocarcinoma cases (clear-cell adenocarcinoma, endometrioid adenocarcinoma, and mucinous adenocarcinoma), $n = 61$. B, Kaplan–Meier curves for overall survival of advanced cases. The cases were analyzed by infiltration of CD8[1] T cells. Total cases, $n = 74$; serous adenocarcinoma cases, $n = 51$; clear-cell adenocarcinoma cases, $n = 11$.
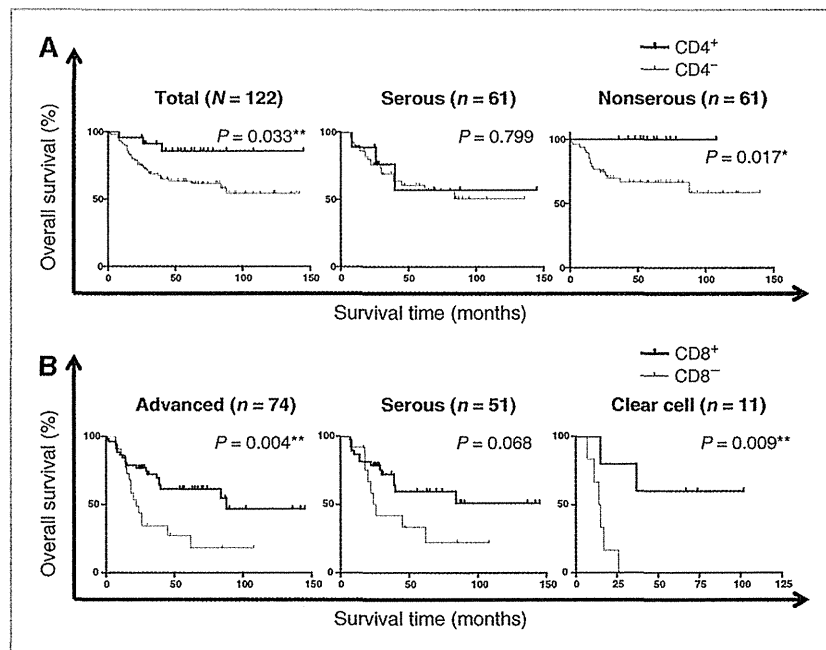
platinum resistance in the study population independent of other clinical characteristic factors (OR, 3.77; 95% CI, 1.08–13.12; $P = 0.046$). Platinum resistance was not correlated with HLA class I expression in the primary tumor site. Because cancer-specific CTLs are primed at regional lymph nodes, we performed immunohistochemical staining of lymph node specimens in lymph node metastasis–positive cases. The expression of HLA class I molecules in lymph nodes was not correlated with the prognosis or platinum resistance (Supplementary Fig. S2 and Supplementary Table S1). Because the differences in chemotherapeutic regimens may have affected these results, we investigated the chemotherapeutic regimens in detail. Most cases (91 of 97 cases) were treated with carboplatin and taxane (paclitaxel, $n = 69$; docetaxel, $n = 22$). There is no difference in TC (paclitaxel and carboplatin) and DC (docetaxel and carboplatin) regimens (Supplementary Fig. S3 and Supplementary Table S2).

Endometrioid histologic subtype showed a significant correlation with good response to platinum agents (OR, 0.22; 95% CI, 0.05–0.97; $P = 0.046$). Clear-cell adenocarcinoma, known to be a platinum-resistant subtype, was excluded from the final model by the stepwise method. This result might have been caused by an insufficient number of advanced clear-cell adenocarcinoma cases.

Discussion

CTLs recognize 8 to 10 amino acid peptide fragments presented by HLA molecules, and play essential role in tumor eradication. However, several mechanisms have been described for human tumor cells that escape from detection and/or destruction by CTLs. (12) Downregulation of HLA class I molecules is one of the major mechanisms that enable tumor cell escape from CTLs, and it is found frequently in solid tumors, including malignant melanoma, breast cancer, stomach cancer, colon cancer, and bladder cancer. The establishment of a novel anti-pan HLA class I mAb (EMR8-5) revealed correlations between HLA class I expression and clinical factors. (21–26).

In this study, we have characterized HLA class I expression as a fine prognosis marker in EOC cases. Several previous reports have shown a correlation between HLA class I downregulation and poor prognosis in EOC. Vitale and colleagues (15) immunostained 51 cases of EOC using anti-HLA class I (HC-10), and anti-TAP1 and -TAP2 antibodies, but there were no statistical correlations between these components and prognosis. Rolland and colleagues (16) report combination staining using HC-10 and β2-microglobulin antibody for 339 cases EOC and suggest that the HC-10$^+$/β2-m$^+$ phenotype is a significant independent prognostic factor. Han and colleagues (17) immunostained components of the antigen-processing machinery (APM), including TAP1, TAP2, tapasin, HLA class I (HC-10), β2-microglobulin, CD3$^+$ T cells, and CD8$^+$ T cells. They report that the downregulation of APM components, a lack of intratumoral T-cell infiltrates, and the suboptimal cytoreduction were independent prognostic factors in multivariate analysis. In all previous studies, only the staining of HLA class I heavy chain with HC-10 was not an independent prognostic marker of EOC. CTLs recognize HLA class I molecules, including HLA-A, HLA-B, and HLA-Cw, and most antigenic peptides are presented by HLA-A (27). However, HC-10 can recognize only HLA-B and HLA-Cw and not HLA-A; EMR8-5

Table 2. Multivariate analysis with Cox proportional hazards model for overall survival

	Total cases (N = 122)				Early cases (n = 48)				Advanced cases (n = 74)			
	Univariate analysis		Multivariate analysis		Univariate analysis		Multivariate analysis		Univariate analysis		Multivariate analysis	
Factors	Risk ratio label (95% CI)	P	Risk ratio label (95% CI)	P	Risk ratio label (95% CI)	P	Risk ratio label (95% CI)	P	Risk ratio label (95% CI)	P	Risk ratio label (95% CI)	P
HLA class I low	2.42 (1.30–4.50)	0.005[b]	2.180 (1.155–4.115)	0.016[a]	7.88 (0.82–76.13)	0.074	7.88 (0.82–76.13)	0.074	2.01 (1.04–3.87)	0.037[a]	1.97 (1.01–3.83)	0.046[a]
No intratumoral T cells (counts <10)												
CD3+	0.82 (0.44–1.52)	0.52			—	—			0.73 (0.38–1.40)	0.33		
CD4+	3.33 (1.03–10.81)	0.045[a]			—	—			2.62 (0.80–8.57)	0.11		
CD8+	1.74 (0.93–3.24)	0.082			4.02 (0.42–38.71)	0.23			2.56 (1.31–4.98)	0.006[b]	2.11 (1.06–4.20)	0.033[a]
Advanced age (over 50 y)	1.78 (0.82–3.87)	0.14			1.84 (0.19–17.74)	0.59			1.38 (0.60–3.16)	0.44		
Multipara (2)	1.78 (0.70–3.24)	0.12			—	—			1.89 (0.83–3.65)	0.14		
Histologic subtype												
Serous	1.55 (0.82–2.92)	0.17			1.15 (0.12–11.10)	0.90			0.67 (0.34–1.32)	0.24		
Cleacell	0.81 (0.40–1.63)	0.55			2.79 (0.29–26.89)	0.37			2.01 (0.91–4.43)	0.083	2.38 (1.03–5.48)	0.042[a]
Endometrioid	0.50 (0.16–1.64)	0.26			—	—			0.68 (0.21–2.24)	0.53		
Mucinous	0.97 (0.23–4.02)	0.97			—	—			1.81 (0.43–7.65)	0.42		
Advanced stage (over FIGO stage III)	8.02 (2.85–22.59)	<0.001[b]			—	—			—	—		
Peritoneal dissemination	4.73 (1.93–9.90)	<0.001[b]			—	—			—	—		
Lymph node metastasis	1.48 (0.78–2.81)	0.23			—	—			—	—		
Platinum resistant	12.46 (5.18–29.96)	<0.001[b]	6.68 (2.56–17.47)	<0.001[b]	—	—			3.93 (1.62–9.54)	0.001[b]	2.25 (0.88–5.75)	0.091
Nonoptimal debulking surgery	7.00 (3.33–14.74)	<0.001[b]	2.93 (1.29–6.63)	0.01[a]	—	—			4.71 (1.83–12.14)	0.001[b]	4.29 (1.55–11.86)	0.005[b]

NOTE: Factors correlated with deciding the FIGO stages were excluded from early/advanced stage table. The other absent columns of early cases are owing to failure of statistical analysis caused by extreme deviation or insufficient number of cases.
[a]$P < 0.05$.
[b]$P < 0.01$.

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Table 3. Multivariate analysis with a logistic regression model of risk for platinum resistance

Factor	Univariate OR (95%CI)	P	Multivariate OR (95%CI)	P
HLA class I low	1.49 (0.55–3.98)	0.43		
Intratumoral T-cell negative (<10)				
CD3[i]	1.03 (0.40–2.65)	0.95		
CD4[i]	2.73 (0.77–9.66)	0.12		
CD8[i]	3.86 (1.15–12.97)	0.029[a]	3.77 (1.08–13.12)	0.037[a]
Advanced age (>50)	—	—		
Multipara (≥ 2)	2.07 (0.79–5.39)	0.13		
Histologic subtype				
Serous	1.08 (0.39–2.98)	0.88		
Clear cell	3.16 (0.63–15.85)	0.16		
Endometrioid	0.21 (0.05–0.89)	0.034[a]	0.22 (0.05–0.97)	0.046[a]
Mucinous	—	—		

NOTE: In advanced mucinous adenocarcinomas, all cases are platinum-resistant and its univariate analysis was failed.
[a]$P < 0.05$.

recognizes HLA-A as well as HLA-B and HLA-Cw (19). Aptsiauri and colleagues (28) categorized HLA loss in human tumor cells into seven phenotypes: total loss, haplotype loss, allelic loss, compound loss unresponsiveness to IFNγ, and aberrant expression of HLA-E with low expression of HLA class I. HC-10 detects total loss and compound phenotypes that lack HLA-B and HLA-Cw as HLA-negative cases, whereas EMR8-5 detects only total loss as HLA-negative cases. Therefore, the differences between our results using EMR8-5 and previous studies using HC-10 might depend on the detection of HLA-A. Our results suggest that EMR8-5 is a better mAb for the evaluation of HLA class I molecules. In addition to HLA class I molecules, APM machinery was evaluated in several previous studies. Tumor-associated antigens or mis-folded defective ribosomal products (DRiP) are degraded into polypeptide fragments by proteasomes in the cytosol. The polypeptide fragments are translocated into the endo-plasmic reticulum (ER) by transporters, TAP1 and TAP2, and loaded onto HLA class I molecules by peptide-loading complexes that are composed from Tapasin, ERp57, and calreticulin. The ER aminopeptidase-1 (ERAP1) generates proper-length peptides (29) for presentation by the HLA class I complex. A previous study reported that downregula-tion of APM was a poorer prognostic factor (17). A lack of APM molecules may lead to reduced expression of MHC class I molecules on the cell surface because there are less antigenic peptide produced for presentation (29). Thus, the reduced HLA class I expression might result from a lack of APM molecule, and is correlated with poorer prognosis. In addition, a lack of some APM molecules has been reported to affect the repertoire of antigenic peptides (30, 31), which, in turn, might be related to decreased recognition by CTLs, resulting in poorer prognosis.

In our study, downregulation of HLA class I was a significant prognostic factor in each disease stage, but infiltration of CD8^{+} T cells showed a significant difference only in advanced stages

of EOC. The latter may be caused by the differing degree of tumor-debulking achievement by surgery. Complete resection of a tumor can be achieved easily when performed at early stages compared with advanced stages. Even if complete resection is achieved macroscopically, the possibility of micro-scopic metastasis may be markedly different between EOC of early and advanced stages. Therefore, in early stages of EOC, influence of the immune-escape mechanism may be less than that in advanced stages. Moreover, advanced EOC that showed antigen-specific T-cell immunity preoperatively may have an advantage over those with poor immune potential. In some cases, we observed dissociation of T-cell infiltration and HLA class I expression. Because T cells need to be activated to infiltrate, the activation status of T cells might be one mech-anism usurped by tumor cells for immune escape. As described previously, tumor cells produce immune-suppressive factors, including VEGFA, TGFβ, and IL10, to suppress the maturation of DCs, which is associated with failure of T-cell activation and abrogation of T-cell infiltration (32). Disruption of T-cell homing is another possible mechanism of tumor cell immune escape. Chemokines, including CCL2, play essential roles in tumor infiltration (33). Thus, the deregulation of chemokine expression and disruption of chemokine signaling might also prevent T-cell infiltration.

Zhang and colleagues (7) report that intratumoral CD3^{+} T-cell infiltration improved the survival of patients with EOC. A significant correlation between CD3^{+} T cells and prognosis was not found in our study (RR, 0.52; 95% CI, 0.44–15.20; P = 0.52; Table 2). Sato and colleagues (20) also report that CD3^{+} T-cell infiltration was not a significant prognostic factor. In the article, they discussed the potential causes of contrary results, and showed that one possibility for the difference may result from the different chemotherapeutic regimens used in the studies. In the study by Zhang and colleagues, patients were treated with a combination of platinum and/or cyclophospha-mide and/or doxorubicin between 1991 and 1995, and they

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were treated with platinum plus paclitaxel between 1995 and 1999 as were patients in this study. All first-line adjuvant chemotherapy was a combination of taxane and platinum agents in our study. Recently, the most frequently used and effective chemotherapeutic regimens for EOC are shifting from the conventional paclitaxel and carboplatin regimen to a set of dose-dense regimens (34), which controls platinum resistance through a novel immune mechanism (35). The authors detected therapeutic effect of the dose-dense chemotherapy that relied on the preservation of treatment-mediated promotion of tumor-specific immunity. Therefore, novel analyses may be needed for novel regimens.

Tregs are characterized as $CD4^+CD25^+FOXP3^+$ T cells that suppress the immune system and the antitumoral immune response of $CD8^+$ T cells (36, 37). Sato and colleagues (20) report that only $CD4^+$ T-cell infiltration was not a poor prognostic factor of EOC but that the CD8:CD4 ratio and CD8:Treg ($CD25^+FOXP3^+$) ratio were significant prognostic factors. In this study, there was no significant difference between the $CD4^+$ T cell–positive and –negative groups with serous histology as reported by Sato and colleagues. However, in the nonserous histologic subtype, $CD4^+$ T-cell positivity was a significant factor of good prognosis in the log-rank test, and all patients in the $CD4^+$ T cell–positive group were alive in the observation period. Furthermore, the high $CD8^+$:Treg ratio showed tendency to be associated with better prognosis than that of low $CD8^+$:Treg ratio cases ($P = 0.089$). The result did not reach statistical significance, but the data suggest the importance of $CD8^+$ T cell:Treg ratio, and our results support those of the previous study.

Shehata and colleagues (18) analyzed the correlation of HLA class I expression and platinum resistance. They showed that HLA class I expression was not a prognostic factor in the platinum-sensitive group but was a prognostic factor in the platinum-resistant group. We performed the same analysis with our data, and found that HLA class I low expression level was not a significant prognostic factor in platinum-resistant cases. In multivariate analysis using a logistic regression model, intraepithelial $CD8^+$ T-cell infiltration was an independent risk factor of platinum resistance in our cases. Endometrioid adenocarcinoma showed an approximately 2-fold lower risk for platinum resistance than other histologic subtypes. Clear-cell adenocarcinoma, which is generally a platinum-resistant subtype, showed no significant difference in this analysis. This may be due to an insufficient number of cases. Clinically, some chemotherapeutic agents show the possibility of overcoming platinum resistance. Gemcitabine, which is commonly used in second-line to third-line chemotherapy for EOC, has been shown to decrease DNA repair in vitro (38). Safra and colleagues (39) report that response rates to the combination of gemcitabine and carboplatin were the same in

platinum-sensitive patients and platinum-resistant patients with recurrent EOC (43.2% vs. 39.1%). Bevacizumab, the first reported effective molecular target agent of ovarian cancer, is expected to be effective for platinum-resistant EOC, and several studies using this agent have been conducted or are ongoing (40). However, its effect is controversial at this time. Our data showed the possibility of a correlation between platinum resistance mechanisms and immune-escape systems. The molecular mechanisms are still elusive; however, there are several hypotheses. Because platinum causes cancer cell death by either apoptosis or necrosis, antigen-presenting cells might acquire tumor-associated antigens expressed in dead cancer cells, resulting in an antitumor immune response. Thus, the clinical response to platinum might be partially due to a secondary immune response, and good immunologic responders might be related to platinum-sensitive cases. Further immunologic analyses are needed.

In summary, our data showed that HLA class I expression detected by EMR8-5 might be a good prognostic marker of EOC and that $CD8^+$ T-cell infiltration in tumors might be a predictive marker of platinum treatment.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

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Production of Multiple CTL Epitopes from Multiple Tumor-Associated Antigens

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Abstract

Identification of antigenic peptides derived from tumor-associated antigens (TAA) enables cancer vaccine therapy using antigenic peptides. Here, we summarize the design of antigenic peptides and induction of cytotoxic T lymphocytes (CTL) using antigenic peptides and validation of CTL.

Key words Tumor-associated antigen, Cytotoxic T lymphocyte, Antigenic peptide, HLA

1 Introduction

In recent years, immunotherapies for malignant diseases have been regarded as the fourth strategy following surgery, chemotherapy, and radiotherapy. The molecular biological characteristics of immunotherapies have been analyzed and have been partially applied in clinical settings. Previous studies showed that antigen-specific immunotherapies such as peptide vaccine therapy were less effective and successful in vivo than in vitro [1, 2]. These results might be due to various escape mechanisms from the immune system, including antigen molecules targeted by immune cells, actions of immune suppression, e.g., regulatory T lymphocytes, or inhibiting cytokines and loss of human leukocyte-associated antigen (HLA) and $\beta2$-microglobulin. It is essential to design antigenic peptides to prevent escape from the immune system [3]. Loss of antigens is thought to be one of the main causes of escape from the immune system, therefore, functional antigens are thought to be suitable targets.

An antigen derived from the melanoma-associated antigen (MAGE) family that was recognized by cytotoxic T lymphocytes from a human melanoma patient was discovered in 1991 [4]. Since then, many tumor-associated antigens (TAA) have been identified and analyzed. Various methods have been used for identifying

candidate TAA, including cDNA expression cloning, cDNA micro-array, DNA subtraction methods, serological identification of antigens by recombinant expression cloning (SEREX methods), and a reverse-immunogenetical approach [4–7]. Although cancer cell-specific proteins are potential immunological targets, it is necessary to determine whether a peptide from a candidate protein can induce a CTL response. In this chapter, we summarize (1) prediction of antigenic peptides, (2) generation of CTL, and (3) validation of CTL and establishment of CTL clones.

2 Materials

2.1 Selection of HLA-Restricted Peptides Derived from Candidate Antigens

1. Putative antigenic peptides can be designed by several website programs (e.g., BIMAS, SYFPEITHI, CTLPred, ProPred1, MAPPP, nHLAPred, LPPEP, SVMHC, NetMHC, MHCPred, Epitope binding, MMPRED, and PREDEP) (Fig. 1) (*see* Note 1) [8, 9].

2. Synthetic peptides.

3. Dimethyl sulfoxide (DMSO).

4. T2 cells cultured in Roswell Park Memorial Institute (RPMI)-1640 supplemented with 10 % FBS (*see* Note 2).

5. Phosphate-buffered saline (PBS) without Ca^{2+} and Mg^{2+} on ice.

6. Opti-MEM® (Life Technologies, Inc., Carlsbad, CA, USA).

7. Anti-HLA-class I monoclonal antibody (mAb) (*see* Notes 3 and 4).

8. ITC-conjugated rabbit antimouse IgG+IgM (KPL, Gaithersburg, MD, USA).

1. Choose HLA allele of your interest

2. Past amino acid sequence of your interest protein

Fig. 1 Representative prediction of antigenic peptides by BIMAS website. BIMAS website: http://www-bimas. cit.nih.gov/molbio/hla_bind/

9. PBS containing 1 % formaldehyde.

10. Disposable pipettes and Pasteur pipettes (sterile).

11. Sterile tubes for flow cytometry.

12. Sterile micropipettors and tips.

13. Centrifuge (refrigerated) with swing-out rotor and appropriate carriers.

14. Hemocytometer and microscope for cell counting.

15. 5 % CO_2 incubator at 26 and 37 °C.

16. Flow cytometer.

2.2 Preparation of APC and CD8⁺ T Cells Isolated from Peripheral Blood Mononuclear Cells (PBMC)

1. Blood sample (see Note 4).

2. Lymphoprep (Nycomed, Oslo, Norway).

3. Anticoagulant agent, e.g., heparin sodium, EDTA, and sodium citrate.

4. PBS with 2 mM EDTA, at room temperature.

5. AIM-V medium.

6. 2-Mercaptoethanol.

7. 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid buffer (HEPES).

8. Human recombinant interleukin-2 (IL-2) (R&D Systems, Minneapolis, MN, USA).

9. Human recombinant interleukin-4 (IL-4) (R&D Systems).

10. Human granulocyte/macrophage-colony stimulating factor (GM-CSF) (R&D Systems).

11. Tumor necrosis factor-α (TNFα) (R&D Systems).

12. Phytohaemagglutinin (PHA-P).

13. MACS separation system (Miltenyi Biotech, Bergisch Gladbach, Germany) using anti-CD8 mAb coupled with magnetic microbeads.

14. Sterile disposable pipettes and Pasteur pipettes.

15. Sterile 50-mL high-clarity polypropylene conical centrifuge tube.

16. Sterile micropipettors and tips.

17. Centrifuge (not refrigerated) with swing-out rotor and appropriate carriers.

18. Sterile 10-cm culture flasks (dish) and 24-well plates.

2.3 Induction of CTL

1. Synthesized peptides dissolved in 20 mg/mL of DMSO.

2. β2-Microglobulin.

3. AIM-V medium supplemented with 10 % human serum, 100 IU/mL of IL-2, 50 μM 2-mercaptoethanol, and HEPES buffer.

4. Human recombinant IL-2 (R&D Systems).

5. Human recombinant interleukin-7 (IL-7) (R&D Systems).

6. Human AB serum.

7. Complete RPMI-1640 medium, i.e., RPMI-1640 supplemented with 10 % fetal bovine serum (FBS).

8. Sterile disposable pipettes and Pasteur pipettes.

9. Sterile 24-well plates.

10. Enzyme-linked immunospot (ELISPOT) Human interferon-γ (IFN-γ) ELISPOT set (Becton Dickinson Biosciences, Franklin Lakes, NJ, USA).

11. Sterile micropipettors and tips.

12. KS ELISPOT assay system (Carl Zeiss, Oberkochen, Germany).

13. Gamma counter (PerkinElmer, Waltham, MA, USA).

14. X-ray irradiation device for cells (SOFTEX, Tokyo, Japan).

2.4 Establishment of CTL Clone

1. CD8 $^+$ T cells (CTL).

2. PBMC from donors.

3. AIM-V medium supplemented with 10 % human serum, 100 IU/mL of IL-2, 50 μM 2-mercaptoethanol, and 10 mM HEPES.

4. Human recombinant IL-2 (R&D Systems).

5. PHA-P.

6. Human AB serum.

7. Sterile disposable pipettes and Pasteur pipettes.

8. Sterile 96-, 48-, and 24-well plates.

9. Sterile micropipettors and tips.

10. X-ray irradiation device for cells (SOFTEX, Tokyo, Japan).

3 Methods

3.1 Selection of HLA-Restricted Peptides Derived from Candidate Antigens

1. Predict putative antigenic peptides with protein sequence (see Note 1). The synthesized peptides should be dissolved in DMSO and stored at -80 °C before use (see Note 2).

2. After incubation of T2 cells in RPMI-1640 culture medium supplemented with 10 % FBS at 26 °C for 18 h, wash the cells with ice-cold PBS (see Note 5).

3. For flow cytometric analysis, divide the cells equally into two sterile tubes and suspend T2 cells with 1 mL of Opti-MEM® with or without 100 μg of peptide, followed by incubation at 26 °C for 3 h and then at 37 °C for 3 h.