drug consisting of calcium folinate, which modulates 5-FU. Therefore, we investigated the safety and immunological responses of personalized peptide vaccination in combination with UFT and LV in 14 patients with metastatic CRC [22]. Peptides were determined based on the presence of peptide-specific CTL precursors and IgG in each patient. A maximum of four peptides was administered weekly with UFT and LV for 4 weeks, followed by the standard 1-week rest period. This therapy was well tolerated, although 1 patient developed a grade 3 skin reaction at the vaccine site. After the tenth vaccination, 9 of 10 patients tested had an increase in peptide-specific interferon-γ production, and 8 of 10 patients tested had an increase in peptide-specific IgG. Six patients had stable disease, and 7 patients had progressive disease, as determined by the RECIST (Response Evaluation in Solid Tumors) criteria. Three of the 6 patients with stable disease showed a minor response; all 3 of these patients showed both strong CTL and IgG responses to at least one of the vaccinated peptides.

Interestingly, IgG responses correlated with overall survival (P= 0.0215) Fig. (1).

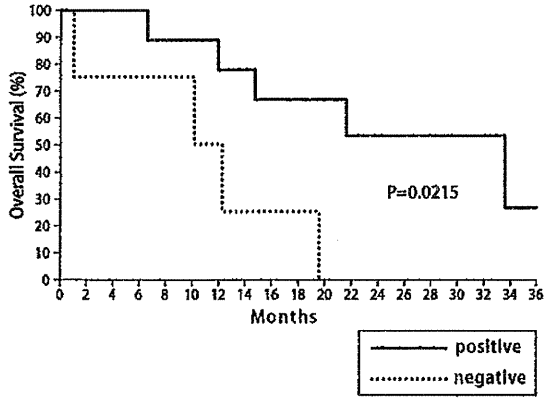

Fig. (1). Correlation between survival and peptide-specific IgG responses.

Overall survival was well correlated with increased levels of peptide-specific IgG (P=0.0215). Solid line: positive peptide-specific IgG response, dotted line: negative peptide-specific IgG response. (Ref. [22])

A similar correlation has been reported for CRC patients receiving a recombinant CEA vaccine [23]. However, the biological roles of IgGs specific to CTL epitopes are unknown. One possibility is that 9-mer peptide-recognizing CD4+ T cells were involved in this phenomenon. Peptides that bind to MHC class II molecules are generally considered to be 12 – 25 amino acids in length; however, the core sites anchored to MHC class II molecules are sufficient even at a length of about nine amino acids [24]. Indeed, our collaborator reported that the 9-mer peptide could induce peptide-specific and HLA-DR-restricted CD4+ T cells [25]. Another possibility is that CD4+ helper T cells might recognize the inoculated peptides presented on the HLA-A24 or -A2 molecules of antigen-presenting cells, resulting in both the activation of helper T cells and the subsequent promotion of IgG production [26]. CD4+ helper T cells are necessary to maintain CD8 T cell immunity [27]. If increased levels of peptide-specific IgGs reflect the activation levels of CD4+ helper T cells, the measurement of peptide-specific IgGs would be worthwhile as an immunological biomarker to predict the clinical benefits of peptide vaccination therapy for cancer patients.

In conclusion, personalized peptide vaccination combined with UFT/LV in patients with metastatic CRC is well tolerated and can induce cellular and humoral immune responses. Increased peptide-specific IgGs may be immunological biomarkers predictive of longer survival. Further trials of these vaccines are merited.

Peptides Derived from Novel Colorectal Cancer-Associated Antigens

cDNA microarray technology coupled with laser microdissection has been used to identify HLA-A24-restricted epitope peptides as potential targets for cancer vaccination in CRC patients [28, 29]. HLA-A24-positive is a dominant population in Japan (approximately 60%), subsequently HLA-A2-positive (approximately 20%). Therefore, to identify the binding epitope to HLA-A24 is essential issue for the successful anti-cancer vaccination in Japan. These antigenic peptides were derived from two different cancer-testis antigens, RNF43 (*ring finger protein 43*) [28] and TOMM34 (34 kDa-translocase of the outer mitochondrial membrane) [29]. Gene expression profiling revealed that RNF43 and TOMM34 were highly expressed in more than 80% of CRC samples, while these transcripts were hardly detectable in normal organs, with the exception of the testis and/or placenta. These peptides could stimulate CTLs that recognized and killed CRC cells. Therefore, RNF43- and TOMM34-derived peptides are promising candidates for the treatment of metastatic CRC. To evaluate the safety and immune response of vaccination with these peptides in combination with oral chemotherapy of UFT and LV for metastatic colorectal cancer, 20 HLA-A2402-positive patients were enrolled in a phase I clinical trial (Okuno *et al.* unpublished data). Eighteen patients were treated with peptides subcutaneously every week and two courses of UFT/LV chemotherapy for 10 weeks. Ten weeks later, the clinical responses were judged by CT scans, and cytotoxic T lymphocyte (CTL) responses against RNF43 and TOMM34 in peripheral lymphocytes were assessed by enzyme-linked immunospot assays. The vaccinations were well tolerated without any serious adverse events. Of the 18 patients, CTL responses were induced against both RNF43 and TOMM34 in 6 patients and against RNF43 or TOMM34 in 9 patients, while 3 patients had no CTL response. The rate of stable disease was 83%, as determined by RECIST criteria. Long-term survivors were observed in the group showing CTL responses against both RNF43 and TOMM34, followed by the group showing CTL responses against only RNF43 or TOMM34. The patients with no CTL responses had the worst survival Fig. (2).

Hazama *et al.* have been investigating a phase I trial of three peptides highly expressed in CRC (RNF43, TOMM34, KOC1), and the epitope peptide of vascular endothelial growth factor receptor 1(VEGFR1), vascular endothelial growth factor receptor 2 (VEGFR2) in combination with

FOLFOX (combination of oxaliplatin, 5-FU, and LV) chemotherapy for metastatic CRC patients. Among 26 patients, 13 patients had a partial response, 12 patients had stable disease, and 1 patient had progressive disease. The median progression-free survival has not been calculated (Shoichi Hazama, personal communication).

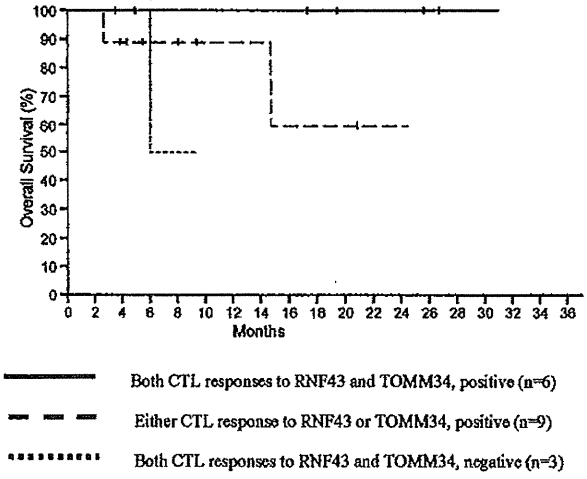

Fig. (2). Overall survival of patients with specific T cell responses to RNF43 and TOMM34.
Patients with responses to both RNF43 and TOMM34 had the best survival. Patients having no responses had worst survival. Patients with one response had intermediate survival.

RATIONALE OF COMBINATION THERAPY

Cancer is an extremely complex and heterogeneous disease that robustly resists host-defense systems and therapeutic efforts. The loss of MHC class I expression is a major mechanism of tumor cell escape from immune surveillance, whereas the appearance of multidrug resistance is the major mechanism of tumor cell resistance to chemotherapy. One approach to overcome the resilience of cancer is the design of a new combination therapy in which each modality imposes independent selective pressure to the acquired mutations of cancer [17].

Chemo-Immunotherapy in CRC

The combined chemo-immunotherapy approach has been criticized on the grounds that chemotherapy is immunosuppressive. This opinion is based on the fact that most cytotoxic drugs can kill granulocyte precursors in bone marrow and thus induce leucopenia, which is associated with the occurrence of bacterial and mycotic infection. However, there is no evidence that cytotoxic chemotherapy affects the antigen-specific CTL response. Recently, Correale *et al.* [30] reported that the antigen-specific killing ability of human CTL lines *in vitro* is not affected by FU, oxaliplatin, or gemcitabine (GEM) if exposure to these drugs does not occur during the stimulation phase. Moreover, they found that chemotherapy (1) up-regulated tumor-associated antigen expression, including CEA or other target molecules such as thymidylate synthase (TS); (2) down-regulated tumor cell resistance to the death signals induced by tumor antigen-specific cytotoxic T lymphocytes; (3) reduced the percentage of PBMCs containing immune-suppressive regulatory T cells (CD4+CD25+T reg) and the number of cells expressing the FAS receptor (CD95); and (4) induced the complete restoration of the CD4/CD8 T cell ratio, which is often reduced in advanced cancer patients showing a progressively deteriorating immune response [31].

HLA Loss or Down-Regulation in Cancer Progression

For successful CTL-based immunotherapy, it is essential to eliminate the loss of major histocompatibility complex (MHC) class I on cancer cells. A large population (30 – 60%) of cancer cells do not express MHC class I molecules, which are crucial for CTL-mediated elimination of cancer cells [32]. This problem, however, could be overcome by the combined use of another type of peptide vaccine, such as peptide of VEGFR1, or VEGFR2 [33], and either chemotherapy [31] or cytokine therapy capable of activating innate immunity including natural killer cells and macrophages. From this viewpoint, the development of an effective vaccine against tumor angiogenesis is suitable, because endothelial cells are genetically stable, do not down-regulate HLA class I molecules, and are critically involved in the progression of a variety of tumors. Furthermore, the CTLs could directly cause damage to the endothelial cells without penetrating any other tissue, and the lysis of even low numbers of endothelial cells within tumor vasculature may result in the destruction of vessel integrity, leading to the inhibition of many tumor cells. The results of a phase I study of multiple peptide vaccination including VEGFR1 and VEGFR2 in combination with FOLFOX chemotherapy for patients with metastatic CRC by Hazama *et al.* are anticipated.

FUTURE PERSPECTIVES

Numerous studies of vaccination in CRC patients have been performed. Antigen-specific responses were induced to some extent, depending on the individual immunizing methods in the trials; however, the clinical responses were marginal. In a meta-analysis by Nagorsen *et al.* [34], the objective response rate was only 0.9% for 527 CRC patients treated with active specific immunotherapy in 32 different studies. There are several possible approaches to improve the poor clinical outcome of vaccine immunotherapy in CRC.

Adjuvant Setting

Despite the nearly complete lack of a clinical response in patients with advanced colorectal cancer, a few studies have shown that adjuvant active specific immunotherapy may be beneficial in subgroups of patients after CRC resection [35, 36]. As we do not expect vaccination in patients with a high tumor burden to be highly clinically effective, we may be able to obtain a better impact on clinical outcome from the adjuvant setting. Recently, we started a randomized trial of CRC-specific peptides (RNF43, TOMM34) in combination with UFT/ LV chemotherapy as adjuvant immunochemotherapy in stage III colorectal cancer patients.

Helper-Peptide Vaccines

Cancer vaccine therapy first focused on the activation of CD8+ cytotoxic T cells (CTLs), which eradicate tumors *in*

vivo. Although many investigators approached this problem by using MHC class I-binding peptides, this approach has been hampered by strong immunosuppression and unknown immune-escape mechanisms in tumor-bearing hosts. CD4+ T cells are crucial for the induction of effective antitumor immunity. In particular, the introduction of T helper type 1 (Th1)-dominant immunity in tumor-bearing hosts is critically important to overcome immunosuppression and induce fully activated tumor-specific CTLs. Nishimura *et al.* [37] reported that adoptively transferred tumor-specific Th1 cells exhibited strong antitumor activity *in vivo*. Moreover, they established cancer-specific helper T cell lines from healthy donors by using cancer antigen NY-ESO-1 derived from overlapping 15-mer synthetic peptides bound to HLA-DR molecules [38]. This method is anticipated to be a new cancer vaccine therapy that elicits a cancer-specific helper T cell response in cancer patients. In collaboration with the Nishimura group, we recently started a helper-peptide vaccine study with Survivin or MAGE-A4 antigen-derived helper peptides for the treatment for advanced CRC patients.

CONFLICT OF INTEREST

None declared.

ACKNOWLEDGEMENT

None declared.

REFERENCES

[1] Boon, T. Tumor antigens recognized by cytolutic T lymphocytes: Present perspectives for specific immunotherapy. *Int. J. Cancer,* **1993**, *54*(2), 177-180.

[2] Rosenberg, S.A. The development of new cancer therapies based on the molecular identification of cancer regression antigens. *Cancer J. Sci. Am.*, **1995**, *1*(2): 89-100.

[3] Jass, J. Lymphocytic infiltration and survival in rectal cancer. *J. Clin. Pathol.*, **1986**, *39*(6), 585-589.

[4] Ropponen, K.M.; Eskelinen, M.J.; Lipponen, P.K.; Alhava,E.; Kosma,V.M. Prognostic value of tumour-infiltrating lymphocytes (TILs) in colorectal cancer. *J. Pathol.*, **1997**, *182*(3), 318-324.

[5] Nanni, O.; Volpi, A.; Frassineti, G.L.; De Paola, F.; Granato, A.M.; Dubini, A.; Zoli, W.; Scarpi, E.; Turci, D.; Oliverio, G.; Gambi, A.; Amadori, D. Role of biological markers in the clinical outcome of colon cancer. *Br. J. Cancer.*, **2002**, *87*(8), 868-875.

[6] Nielsen, H.J.; Hansen, U.; Christensen, I.J.; Reinert, C.M.; Brunner, N.; Moesgaard, F. Independent prognostic value of eosinophil and mast cell infiltration in colorectal cancer tissue. *J. Pathol.*, **1999**, *189*(4), 487-495.

[7] Roncucci, L.; Fante, R.; Losi, L.; Di Gregorio, C.; Micheli, A.; Benatti, P.; Madenis, N.; Ganazzi, D.; Cassinadri, M.T.; Lauriola, P.; Ponz de Leon, M. Survival for colon and rectal cancer in a population-based cancer registry. *Eur. J. Cancer,* **1996**, *32A*(2), 295-302.

[8] Naito, Y.; Saito, K.; Shiiba, K.; Ohuchi, A.; Saigenji, K.; Nagura, H.; Ohtani, H. CD8+ T cells infiltrated within cancer cell nests as a prognostic factor in human colorectal cancer. *Cancer Res.*, **1998**, *58*(16), 3491-3494.

[9] Funada, Y.; Noguchi, T.; Kikuchi, R.; Takeno, S.; Uchida, Y.; Gabbert, H.E. Prognostic significance of CD8+ T cell and macrophage peritumoral infiltration in colorectal cancer. *Oncol. Rep.*, **2003**, *10*(2), 309-313.

[10] Watanabe, T., Wu, T.T.; Catalano, P.J; Ueki, T.; Satriano, R.; Haller, D.G.; Benson, A.B.; Hamilton, S.R. Molecular predictors of survival after adjuvant chemotherapy for colon cancer. *N. Engl. J. Med.*, **2001**, *344*(16), 1196-1206.

[11] Liu, K.; Wang, C.; Chen, L.; Cheng, A.; Lin, D.; Wu, Y.; Yu, W.; Hung, Y.; Yang, H.; Juang, S.; Peng, J. Generation of carcinoembryonic antigen (CEA)-specific T cell responses in HLA-A0201 and HLA-A2402 late-stage colorectal cancer patients after vaccination with dendritic cells loaded with CEA peptides. *Clin. Cancer Res.*, **2004** *10*(8), 2645-2651.

[12] Weihrauch, M.R.; Ansen, S.; Jurkiewicz, E.; Geisen, C.; Xia, Z.; Anderson, K.S.; Gracien, E.; Schmidt, M.; Wittig, B.; Diel, V.; Wolf, J.; Bohlen, H.; Nadler, L.M. Phase I/II combined chemoimmunotherapy with carcinoembryonic antigen-derived HLA-A2-restricted CAP-1 peptide and irinotecan, 5-fluorouracil, and leucovorin in patients with primary metastatic colorectal cancer. *Clin. Cancer Res.*, **2005**, *11*(16), 5993-6001.

[13] Kavanagh, B.; Ko, A.; Venook, A.; Margolin, K.; Zeh, H.; Lotze, M.; Schillinger, B.; Liu, W.; Lu, Y.; Mitsky, P.; Schilling, M.; Bercovici, N.; Loudovaris, M.; Guillermo, R.; Lee, S.M.; Bender, J.; Mills, B.; Fong, L. Vaccination of metastatic colorectal cancer patients with matured dendritic cells loaded with multiple major histocompatibility complex class I peptides. *J. Immunother.*, **2007**, *30*(7): 762-772.

[14] Marshall, J.L.; Hawkins, M.J.; Tsang, K.Y.; Richmond, E.; Pedicano, J.E.; Zhu, M.Z.; Schlom, J. Phase I study in cancer patients of a replication-defective avipox recombinant vaccine that expresses human carcinoembryonic antigen. *J. Clin. Oncol.*, **1999**, *17*(1), 332-337.

[15] Marshall, J.L.; Gully, J.L.; Arlen, P.M., Beetham, P.K.; Tsang, K.; Slack, R.; Hodge, J.W.; Doren, S.; Grosenbach; D.W.; Hwang, J.; Fox, E.; Odogwu, L.; Park, S.; Panicali, D.; Schlom, J. Phase I study of sequential vaccinations with fowlpox-CEA(6D)-TRICOM alone and sequentially with vaccinia-CEA(6D)-TRICOM, with and without granulocyte-macrophage colony-stimulating factor, in patients with carcinoembryonic antigen-expressing carcinomas. *J. Clin. Oncol.*, **2005**, *23*(4), 720-731.

[16] Rosenberg, S.A.; Yang, J.C.; Restifo, N.P. Cancer immunotherapy: moving beyond current vaccines. *Nat. Med.*, **2004**, *10*(9), 909-915.

[17] Itoh, K.; Yamada, A. Personalized peptide vaccines: a new therapeutic modality for cancer. *Cancer Sci.*, **2006**, *97*(10), 970-976.

[18] Sato, Y.; Maeda, Y.; Shomura, H.; Sasatomi, T.; Takahashi, M.; Une, Y.; Kondo, M; Shinohara, T.; Hida, N.; Katagiri, K.; Sato, M.; Yamada, A.; Yamana, H.; Harada, M.; Itoh, K.; Todo, S. A phase I trial of cytotoxic T-lymphocyte precursor-oriented peptide vaccines for colorectal carcinoma patients. *Br. J. Cancer.*, **2004**, *90*(7), 1334-1342.

[19] Noguchi, M.; Itoh, K.; Suekane, S.; Morinaga, A.; Sukehiro, A.; Suetsugu, N.; Katagiri, K.; Yamada, A.; Noda, S. Immunological monitoring during combination of patient-oriented peptide vaccination and estramustine phosphate in patients with metastatic hormone refractory prostate cancer. *Prostate,* **2004**, *60*(1), 32-45.

[20] Miyagi, Y.; Imai, N.; Sasatomi, T.; Yamada, A.; Mine, T.; Katagiri, K.; Nakagawa, M.; Muto, A.; Okouchi, S.; Isomoto, H.; Shirouzu, K.; Yamana, H.; Itoh, K. Induction of cellular immune responses to tumor cells and peptides in colorectal cancer patients by vaccination of SART3 peptides. *Clin. Cancer Res.*, **2001**, *7*(12), 3950-3962.

[21] Sato, Y.; Fujiwara, T.; Mine, T.; Shomura, H.; Homma, S.; Maeda, Y.; Tokunaga, N.; Ikeda, Y.; Ishihara, Y.; Yamada, A.; Tanaka, N.; Itoh, K.; Harada, M.; Todo, S. Immunological evaluation of personalized peptide vaccination in combination with a 5-fluorouracil derivative (TS-1) for advanced gastric or colorectal carcinoma patients. *Cancer Sci.*, **2007**, *98*(7), 1113-1119.

[22] Hattori, T.; Mine, T.; Komatsu, N.; Yamada, A.; Itoh, K.; Shiozaki, H.; Okuno, K. Immunological evaluation of personalized peptide vaccination in combination with UFT and UZEL for metastatic colorectal carcinoma patients. *Cancer Immunol. Immunother.*, **2009**, *58*(11), 1845-1854.

[23] Ullenhag, G.J.; Frödin, J.E.; Jeddi-Tehrani, M.; Strigard, K.; Eriksson, E.; Samanci, A.; Choudhury, A.; Nilsson, B.; Rossmann, E.D.; Mosolits, S.; Mellstedt, H. Durable carcinoembryonic antigen (CEA)-specific humoral and cellular immune responses in colorectal carcinoma patients vaccinated with recombinant CEA and granulocyte/macrophage colony-stimulating factor. *Clin. Cancer Res.*, **2004**, *10*(10), 3273-3328.

[24] Rammensee, H.G.; Frige, T.; Stevanovic, S. MHC ligands and peptide motifs. *Immunogenetics,* **1995** *41*(4), 178-228.

[25] Harada, M.; Gohara, R.; Matsueda, S.; Muto, A.; Oda, T.; Iwamoto, Y.; Itoh, K. *In vivo* evidence that peptide vaccination can induce HLA-DR-restricted CD4+ T cells reactive to a class I tumor peptide. *J. Immunol.*, **2004**, *172*(4), 2659-2667.

[26] Mine, T.; Sato, Y.; Noguchi, M.; Sasatomi, T.; Gouhara, R.; Tsuda, N.; Tanaka, S.; Shomura, H.; Katagiri, K.; Rikimaru, T.; Shichijo, S.; Kamura, T.; Hashimoto, T.; Shirouzu, K.; Yamada, A.; Todo, S.; Itoh, K.; Yamana, H. Humoral responses to peptides correlate with overall survival in advanced cancer patients vaccinated with peptides based on pre-existing, peptide-specific cellular responses. *Clin. Cancer Res.*, **2004**, *10*(3), 929-937.

[27] Antony, P.A.; Piccirillo, C.A.; Akpinarli, A.; Finkelstein, S.E.; Speiss, P.J.; Surman, D.R.; Palmer, D.C.; Chan, C.C. Klebanoff, C.A.; Overwijk, W.W.; Rosenberg, S.A.; Restifo, N.P. CD8+ T cell immunity against a tumor/self-antigen is augmented by CD4+ T helper cells and hindered by naturally occurring T regulatory cells. *J. Immunol.* **2005**, *174*(5), 2591-2601.

[28] Uchida, N.; Tsunoda, T.; Wada, S.; Furukawa, Y.; Nakamura, Y.; Tahara, H. Ring Finger protein 43 as a new target for cancer immunotherapy. *Clin. Cancer Res.*, **2004**, *10*(24), 8577-8586.

[29] Shimokawa, T.; Matsushima, S.; Tsunoda, T.; Tahara, H.; Nakamura, Y.; Furukawa, Y. Identification of TOMM34, which shows elevated expression in the majority of human colon cancers, as a novel drug target. *Int. J. Oncol.*, **2006**, *29*(2), 381-386.

[30] Correale, P.; Del Vecchio, M. T.; Genova, G. D.; Savellini, G. G.; Placa, M.L.; Terrosi, C.; Vestri, M.; Urso, R.; Lemonnier, F.; Aquino, A.; Bonmassar, E.; Giorgi, G.; Francini, G.; Cusi, M.G. 5-fluorouracil-based chemotherapy enhances the antitumor activity of a thymidylate synthase-directed polyepitopic peptide vaccine. *J. Natl. Cancer Inst.* **2005**, *97*(19), 1437-1445.

[31] Correale, P.; Cusi, M.G.; Tsang, K.Y.; Del Vecchio, M.T.; Marcili, S.; Placa, M.L.; Intrivici, C.; Aquino, A.; Micheli, L.; Nencini, C.; Ferrari, F.; Giorgi, G.; Bonmassar, E.; Franoini, G. Chemoimmunotherapy of metastatic colorectal carcinoma with gemcitabine plus FOLFOX 4 followed by subcutaneous granulocyte macrophage colony-stimulating factor and interleukin-2 induces strong immunologic and antitumor activity in metastatic colon cancer patients. *J. Clin. Oncol.*, **2005**, *23*(35), 8950-8958.

[32] Janeway, C.A.; Sclomchik, M.J.; Travers, P.; Walport, M. Using the immune response to attack tumors. In: *Immunobiology, 6th ed.*; Janeway, C.A.; Sclomchik, M.J.; Travers, P.; Walport, M., Eds.; Garland Science: New York, **2004** pp.630-642.

[33] Wada, S.; Tsunoda, T.; Baba, T.; Primus, F.J.; Kuwano, H.; Shibuya, M.; Tahara, H. Rationale for antiangiogenic cancer therapy with vaccination using epitope peptides derived from human vascular endothelial growth factor receptor 2. *Cancer Res.*, **2005**, 65(11), 4939-4946.

[34] Nagorsen, D.; Thiel, E. Clinical and immunologic responses to active specific cancer vaccines in human colorectal cancer. *Clin. Cancer Res.*, **2006**, *12*(10): 3064-3069.

[35] Vermorken, J.B.; Claessen, A.M.; van Tinteren, H.; Gall, H.E.; Ezinga, R.; Meijer, S.; Scheper, R.J.; Meijer, C.J.; Bloemena, E.; Ransom, J.H.; Hanna, M.G.Jr.; Pinedo, H.M. Active specific immunotherapy for stage II and stage III human colon cancer: a randomised trial. *Lancet*, **1999**, *353*(9150), 345-350.

[36] Harris, J.E.; Ryan, L.; Hoover, H.C.,Jr.; Stuart, R.K.; Oken, M.M.; Benson, A.B.3rd.; Mansour, E.; Haller, D.G.; Manola, J.; Hanna, M.G.Jr. Adjuvant active specific immunotherapy for stage II and III colon cancer with an autologous tumor cell vaccine: Eastern Cooperative Oncology Group Study E5283. *J. Clin. Oncol.*, **2000**, *18*(1): 148-157.

[37] Chamoto, K.; Wakita, D.; Narita, Y. Zhang, Y.; Noguchi, D.; Ohnishi, H.; Iguchi, T.; Sakai,T.; Ikeda, H.; Nishimura, T. An essential role of antigen-presenting cell/T-helper type 1 cell-cell interactions in draining lymph node during complete eradication of class II-negative tumor tissue by T-helper type 1 cell therapy. *Cancer Res.*, **2006**, 66(3): 1809-1817.

[38] Ohkuri, T.; Sato, M.; Abe, H.; Tsuji, K.; Yamagishi, Y.; Ikeda, H.; Matsubara, N.; Kitamura, H.; Nishimura, T. Identification of a novel NY-ESO-1 promiscuous helper epitope presented by multiple MHC class II molecules found frequently in the Japanese population. *Cancer Sci.*, **2007**, *98*(7): 1092-1098.

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EUROPEAN JOURNAL OF CLINICAL & MEDICAL ONCOLOGY REVIEW ARTICLE

Intracellular Glutathione in Monocytes are Useful Biomarker of Immune Status of Tumor Bearing Patients

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ABSTRACT

Solid tumor patients, with low immune response, tend to have poor survival outcome after surgery. A new concept has emerged that preoperative determination of patient's immune status will be helpful in determining appropriate therapeutic options to achieve the best possible postoperative outcome for solid tumor bearing patients. Here we propose a method of predicting patient's immune status using peripheral blood specimen. This method is based on the finding that a relationship exists among preoperative monocytes (Mo) status (reductive, R-Mo/oxidative, O-Mo) determined by glutathione levels, presence/absence of tumor infiltrating lymphocytes (TIL) in tumor parenchyma, and the patient's survival after operation. In fact, R-Mo with a higher icGSH can stimulate $CD4^+$ T-cells to produce IFN-γ more effectively than O-Mo and thereby induce antitumor Th1 responses. Based on this concept, we will in this review introduce a method of predicting local antitumor reactions in preoperative solid tumor patients using peripheral blood specimen. In patients who were predicted as having poor immune response, we will overview the probability of inducing antitumor immune response, before patients undergo surgery, by converting O-Mo to R-Mo using biological response modifiers (BRM) such as lentinan. These clinically practical methods can be helpful in determining appropriate therapeutic options to achieve the best possible postoperative outcome for solid tumor bearing patients.

Keywords: glutathione, reductive monocyte, oxidative monocyte, tumor infiltrating T-cell, biological response modifier

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INTRODUCTION

In this brief review we will introduce a new concept of regulating host response prior to surgery, leading to the proposal of a clinically practical method to improve the survival rate of patients with solid tumors using individualized therapy. We will explore the possibility of predicting local antitumor reactions without surgery using biomarkers easily measurable in peripheral blood specimens. Such predictions can be helpful in determining appropriate therapeutic strategies before surgery to achieve the best possible postoperative outcome for solid tumor bearing patients.

Many previous reports have demonstrated the critical role of tumor microenvironments, especially the presence of TIL in tumor stroma, for a good prognosis of solid tumor bearing patients.[1–3] The presence of TIL is usually determined in resected tumors using pathological paraffin sections. Patients with poor antitumor immunity are usually unable to overcome the risk of micrometastasis associated with surgery and, therefore, tend to have poor survival outcome. Predicting antitumor immune response at local tumor growing sites using only peripheral blood specimens enables us to classify the potential clinical outcome of concerned patients based on the presence or absence of antitumor immune response in their local tumor growing sites. This presurgical classification can enable doctors to administer individualized presurgery treatment to patients with limited antitumor immune response. Presurgical determination of antitumor immune responses allows for patient-oriented therapeutic options and may result in better survival outcome of patients. Several treatments such as BRM,[4,5] low dose chemotherapy, or radiation therapy[6,7] are known to be effective at inducing antitumor immune response.

In a prior report, we demonstrated that determining icGSH redox status in peripheral Mo correlated with the existence of TIL in tumor parenchyma.[8] In this review, we will focus on the relationship between antitumor activity and intracellular redox status of peripheral Mo and discuss the following issues: (1) the relationship between icGSH levels of peripheral Mo and antitumor activity, (2) the possibility of using BRM to modulate intracellular redox status of peripheral Mo in order to induce adequate antitumor immune response in solid tumor bearing patients.

A FUNCTIONAL HETEROGENEITY OF MO AND ANTITUMOR IMMUNE-REACTION IN SOLID TUMORS

To induce the efficient sensitization of immune cells with solid tumors, helper T-cells, and CTL are required to transmigrate the endothelium and tumor stromal tissues and penetrate the tumor parenchymal area.[9] In our previous study, we reconciled the relationship among the pathology of the tumor peri-stroma area, the existence of TIL in tumor parenchymas, and the GSH status of peripheral Mo.[8,10]

Many researchers consider Mϕ as suppressors of antitumor immune response, however, we will shed light on the functional heterogeneity of Mϕ. Further, many reports have mentioned Mϕ/Mo plasticity and their ability to change their status based on environmental factors such as cytokines, microbial stimuli, and hypoxia. Hamuro et al previously reported that Mϕs were divided into at least two activated states based on their icGSH content. Mϕs with elevated icGSH are arbitrarily termed as R-Mϕ and those with reduced icGSH as O-Mϕ.[11] They suggest that each of these two types of Mϕ has its own distinct function; R-Mϕ with high icGSH induces the polarization to the Th1 response and O-Mϕ with reduced levels of icGSH induces the Th2 response. This means that Th1/Th2 balance is regulated by the balance between R-Mo/Mϕ and O-Mo/Mϕ. The balance between these two classes of Mϕ is associated with the skewed production levels of IL-12 and IL-10, which further affects the development of the tumor stroma area, possibly due to the skewed production of IFN-γ. Peterson et al also demonstrated that GSH levels in antigen-presenting cells modulate Th1 and Th2 response patterns.[12]

Recently, analogous to the Th1/Th2 nomenclature, a dichotomy of polarized M1- and M2-Mϕ has been proposed.[13,14] The M1-Mϕ are characterized as producing high levels of IL-12 and low levels of IL-10, while M2-Mϕ produce low levels IL-12 and have the tendency to shift from arginine metabolism to the production of ornithine and polyamines via arginase, as was previously shown by Hamuro et al[11] and Murata et al.[15,16] Many circumstantial evidence have suggested that M1-Mϕ closely corresponds to R-Mo/Mϕ and M2 Mϕ to O-Mϕ.

While the classification of the propensities of Mϕ as R-Mϕ and O-Mϕ based on the intracellular content of GSH may not be absolute but rather relative, we adopted this method as we think it is useful in conceptualizing the convergence of diversified immunological outcomes initiated by a variety of stimuli. The R-Mϕ and O-Mϕ classification simply refers to distinct metabolic states or to the intracellular redox status of Mϕ; however, this status may actually exist on a continuum and be distinctly separable. The R-Mo/O-Mo paradigm is summarized in Figure 1.

Mϕ/Mo REDOX STATUS AND THE ROLE OF icGSH

The GSH is a nonprotein tripeptide that contains thiol. It is abundant in virtually all cells, playing significant roles in many biological processes. GSH also constitutes the first line of cellular defense mechanisms against oxidative injury and is the major intracellular redox buffer in ubiquitous cell types. In this way icGSH/GSSG dominates the generation of diverse cytokines and inflammatory mediators. Hamuro et al[11] have shown that GSH redox status plays a central role in determining which of the R- and O-Mϕ/antigen presenting cells (APC) are dominant during a variety of immune responses. They demonstrated that ic-GSH/GSSG dominates the generation of diverse cytokines and inflammatory mediators. Redox status in cytosole, such as changes in the amount of GSH (or the GSH/GSSG ratio), affects NF-ϕB/IϕB complex leading to its activation, NF-ϕB then translocates into the nucleus, resulting in DNA binding and transactivation.

Utsugi et al[17,18] revealed the molecular mechanism by which GSH redox status affects IL-12 production in human APC. They demonstrated that p38 mitogen-activated protein kinase (MAPK) positively, and c-jun N-turminal kinase (JNK) negatively regulates LPS-induced IL-12 production and, as a result, the GSH/GSSG ratio induced by GSH precursor enhances IL-12 production.

In a recent report Alam et al[19] demonstrated that an altered cellular redox plays a profound role in inflammation by activating various kinases and redox-sensitive transcription factors such as NF-κB rel proteins. NF-κB rel proteins differentially regulate the genes encoding various proinflammatory cytokines. The GSH/GSSG balance modulates IκBα signaling and the levels of CaM expression in Mϕ, which subsequently influences nuclear c-rel translocation, and thereby leads to the regulation of IL-12 production levels.

Further, low icGSH levels in APC are correlated with defective processing of antigen with disulfide bonds, indicating that this thiol may be a critical factor in regulating antigen processing.[20,21]

RELATIONSHIP BETWEEN R-Mo/O-Mo STATUS AND ANTITUMOR IMMUNITY

In a prior report we applied the R-Mo/O-Mo parameter to clinical specimens and determined the GSH status in Mo from 30 colon/rectal cancer-bearing patients (Stage O-IV) prior to surgery. The GSH index is useful for measuring individual patient's icGSH as it requires a relatively small amount of Mo compared with other biochemical measurements. The Mo were stained with monochlorobimane and observed with a fluorescence microscope.[8] Each patient's Mo image was analyzed using image software and quantified as total GSH in the $CD14^+$ Mo (designated as GSH index). The Cox's proportional analysis of the 30 patients showed that GSH index and TIL presence/absence were significant independent factors that could predict survival. These results indicate that knowing R-Mo or O-Mo status is as equally predictive of patient survival as knowing whether TIL is present or absent in tumor parenchyma. Therefore determining R-Mo or O-Mo status is a useful biomarker to understand and predict the antitumor status of solid tumor bearing patients.

The evidence from histology and survival of colorectal patients described above suggest that antitumor responses occur at a high frequency in patients with R-Mo. In a previous paper, we demonstrated that IL-12 responsiveness was a significant factor that predicts survival and is a good indicator that allowed us to predict the existence of sensitized T-cells as part of an ongoing antitumor immune response.[8] When comparing R-Mo colorectal cancer patients, patients showing TIL presence, and those with IL-12 responsiveness, the

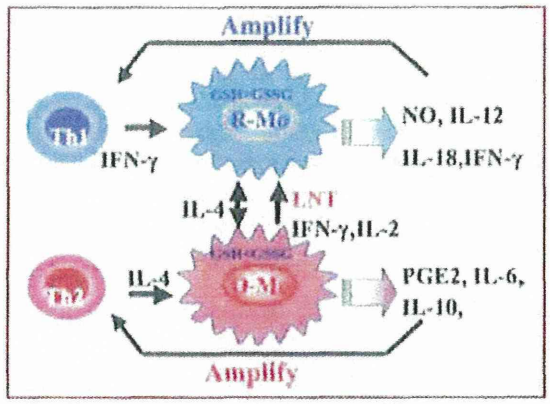

Figure 1. This diagram shows the phase transitions of O-Mo to R-Mo and vice versa. Mo/Mϕ were divided into two activated status based on the intracellular content of GSH. IcGSH in Mo/Mϕ is critical for the secretion of not only various cytokines but also various effecter molecules. These factors further indicate a crucial role of intracellular GSH in Mo/Mϕ in determining whether Th1 or Th2 response is dominant. It is possible to transform O-Mo to R-Mo by administering OK-432 and LNT.

relationship is such that the R-Mo group and TIL presence group mostly overlap but not completely, whereas the IL-12 responsiveness group is contained within the range of the other two groups (Figure 2).

These results demonstrate that the intracellular redox status monitored by icGSH levels in Mo/Mϕ is pivotal for the development of antitumor response (presumably Th1 and wound healing status); therefore, determining icGSH has great potential as a favorable prognostic biomarker in predicting survival. Additionally, there seems to be a significant advantage in using GSH index to classify cancer stage to predict overall survival. Recently, Ma et al[22] demonstrated that the presence of the M1 form of tumor-associated Mϕ in resected tumor specimens was positively associated with the survival of lung cancer patients. These results confirm the idea that the microenvironment supported by R-Mϕ /M1-Mϕ, lead to patients' Th1 antitumor immune response and affect their prognosis.

There are several reports that activated Mϕ/Mo icGSH levels are higher than resident Mϕ/Mo.[23] Furthermore, the capacity of allo-stimulatory and IFN-γ production correlated with icGSH levels in Mo-derived dendritic cells.[24] Low icGSH levels in antigen-presenting cells correlated with defective processing of antigens.[21] Furthermore, GSH and IL-2 are involved in the growth and replication of activated lymphocytes.[25] These data suggest that the icGSH levels of Mo/Mϕ/DC affect not only antitumor activity, but also the antigen-presenting activities of Mo/DC. Additionally, it is easy to conclude that the icGSH of Mo influences lymphocyte proliferation, differentiation, movement, and chemokine/cytokine production.[26–28]

PHASE TRANSITION OF Mϕ STATUS TO INDUCE ANTITUMOR IMMUNE RESPONSE

As we have prior stated, intracellular redox status of Mϕ is critical for inducing antitumor responses. Functional plasticity of Mϕ offers hope that phase transition of Mϕ status from O-Mϕ to R-Mϕ may act positively to induce antitumor immune response. Murata et al[15] confirmed that the GSH content in Mϕ could be modulated easily by the application of N-acetyl cystein (NAC), glutathione monoester (GSH-OEt), buthionin sulfoximine (BSO), and maleic acid diethyl ester (DEM) in vitro and in vivo. As a result, GSH deprived Mϕ (O-Mϕ) showed elevated IL-6 and IL-10 production. On the

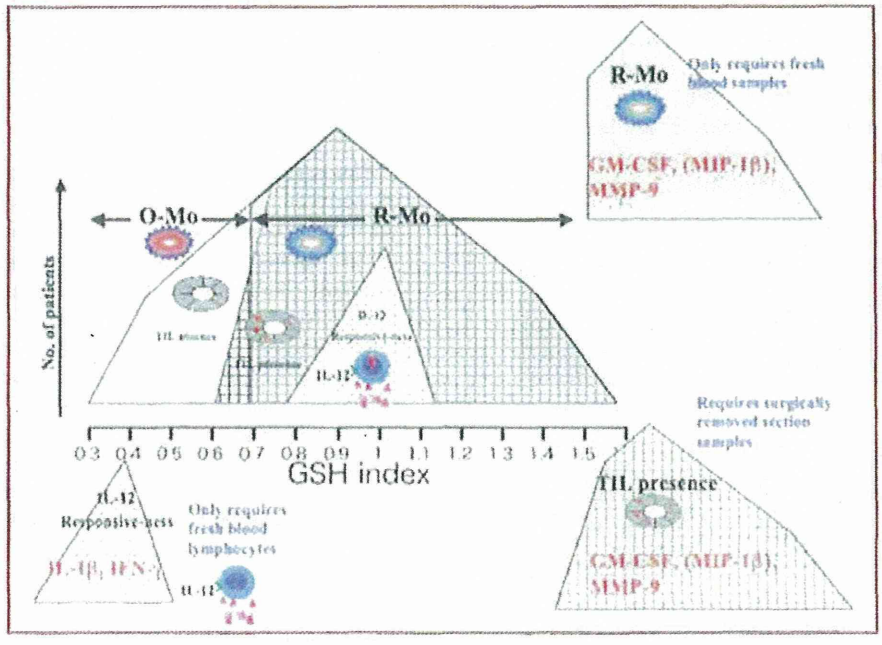

Figure 2. Schematic diagram of the relationship among Mo status based on the GSH index, TIL presence/absence in tumor parenchyma, and IL-12 responsiveness of CD4T and/or CD8T-cells.

other hand, GSH-OEt primed R-Mϕ produced IL-12 upon IFN-γ and LPS stimulation, whereas DEM elicited O-Mϕ did not. These results reveal that various appropriate treatments enable Mϕ phase transition and induce Th1 polarization.

Lentinan and OK432 are commonly used clinically in Japan, and we believe their effects are partly dependent on the transition of O-Mo to R-Mo and determine the Mo status of BRM treated patients. As shown in **Figure 3**, it is possible for O-Mo to transit to R-Mo by clinical treatments using LNT or OK432 agents.

The LNT is a β-(1–3)-D-glucan extracted from edible mushroom Lentinus edodes (Berk.) Sing and then purified.[4] β-(1–3)-D-glucans, a physiological molecule ubiquitously present in the cell walls of fungi, are well known as being critical for the host defense system.[29] Pre- and postoperative administration of β-D-(1–3) glucan, LNT, in combination with interleukin-2 (IL-2) was reported to completely cure murine tumor models due to the synergistically augmented tumor tissue stromal reaction. LNT-induced R-Mϕ induce Th1 response by producing IL-12, and reducing O-Mϕ induced Th2 thereby reducing the production of IL-6, IL-10, and prostaglandin (PG) E2. In patients with inoperable recurrent gastric cancer undergoing chemotherapy, intravenously injected LNT, because of its ability to induce Th1 response through its action on monocytes, elicits a remarkable life-prolonging effect and is now widely used as an immunotherapeutic drug.[30]

Our previous reports showed that OK-432 administration increased the GSH indices of Mo, and these Mo stimulated CD4T cells to produce more IFN-ϕ than O-Mo.[8] Streptococcal preparation OK-432, a penicillin-killed and lyophilized preparation from the low-virlence strain (Su) of Streptococcus pyogenes was developed by Okamoto et al[31] and is commonly used as a BRM for immunotherapy in malignant tumors. It has been shown that the lipoteichoic-acid-related molecule (OK-PSA), extracted from OK-432, induced Th1-type cytokines, such as IFN-γ, TNF-α, IL-2, IL-12, and IL-18 and elicited a marked antitumor effect.[32] Additionally, in vitro culture of immature DC generated from adherent peripheral blood mononuclear cells using GM-CSF and IL-4 with OK432 resulted in increased cell surface expression of matured DC marker (OK-DC). Prior reports have demonstrated that OK432, a strong inducer of IL-12 and IFN-γ, efficiently augment the primary allogeneic T-cell responses and cytotoxic T lymphocytes (CTL) response by OK-DC. These findings strongly suggest that for cancer patients with a Th2-dominant state, OK-432 is a useful immunotherapeutic agent, since it acts as a potent Th1 response inducer by converting Mo status.

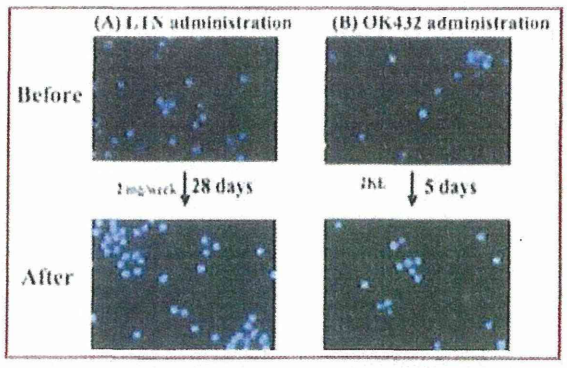

Figure 3. Conversion of O-Mo to R-Mo after BRM treatment. MCB staining in two cancer patients treated with LTN or OK-432. Left panels: Mo from lung cancer patient before and after treatment with LTN. Right panel: Mo from breast cancer patient before and after treatment with OK432.

ALTERNATIVE BIOMARKER TO GSH MO

We have demonstrated previously that GSH index obtained from peripheral blood specimen is an alternative biomarker to confirm TIL existence in tumor parenchyma. IL-12 responsiveness using purified blood CD4/CD8 T lymphocytes from colon/rectal cancer patients, suggests the existence of sensitized CD4/CD8 T-cells.[8,33] Albeit being good biomarkers to predict antitumor immune-reactions in solid tumor bearing patients without the need of surgery, both GSH index and IL-12 responsiveness require fresh blood lymphocytes/Mo. Additionally, in our prior experiments, we pursued alternative biomarkers using plasma cytokine/chemokine analysis with a suspension array system using the original 30 colon/rectal cancer patients. We found that several cytokine/chemokine, and MMP levels in the preoperative plasma from colorectal cancer patients correlated well with Mo redox status, TIL, and overall survival (**Table 1**). Higher GM-CSF was also observed in the plasma of the R-Mo and TIL presence group, and ultimately these groups experienced good survival outcome. Although the difference was marginal, higher IL-1β and MIP-1β levels were found in the R-Mo or TIL presence group and this led to good survival outcome. Higher plasma MMP-9 was also observed in the R-Mo and TIL presence group. Additionally, higher plasma IL-1β and IFN-γ correlated with positive IL-12 responsiveness of CD4/CD8 T-cells and also led to good survival outcome. As shown in **Table 1**, these results indicate that GM-CSF, MIP-1β, and MMP-9 are biomarkers for R-Mo and TIL existence, and IL-1β and IFN-ϕ are related to antitumor T-cell responses.

Why does higher plasma levels of GM-CSF, IL-1β, and MIP-1β correlate with R-Mo/TIL existence in tumor parenchyma? GM-CSF is well known as a key cytokine for the maturation of DC derived Mo. MIP-1β is a chemokine, which acts as a potent chemoattractant for immune cells such as CD4T, CD8T, NK, and DC cells. It has been demonstrated that intratumoral expression of MIP-1ϕ induces strong antitumor responses.[34,35] Besides its central role in inflammation, IL-1β has also been recognized as a powerful player in tumor progression, angiogenesis, and invasiveness. On the other hand, IL-1β also has been used as an adjuvant to mount antitumor immunity.[36,37] Although the role of MMP-9, a member of gelatinase subgroup, in cancer is controversial, a report has suggested that high MMP-9 in epithelial ovarian cancer cells was associated with long-term survival.[38]

When R-Mϕ is dominant in tumor tissue stromal inflammation, tissues are remodeled after extravasation occurs,

Table 1. Relationship Among Mo Status, TIL Existence, IL-12 Responsiveness and Overall Survival

Categories									Cox Hazard Analysis	
	Pre-operative Mo Status			TIL in Tumor Islets			IL-12 Responsiveness		Overall Survival	
Plasma Cytokine log (pg/ml)	O-Mo (n=6)	R-Mo (n=14)	p	absence (n=7)	existence (n=23)	p	positive (n=7)	negative (n=23)	p	HR (95% CI)	p
O-Mo/R-Mo	—	—	—	—	—	—	—	—	—	0.194 (0.038–0.887)	0.0352*
absence/existance TIL	—	—	—	—	—	—	—	—	—	0.194 (0.038–0.887)	0.0352*
IL-12 responsiveness	—	—	—	—	—	—	—	—	—	$1.86e^{-7}$ (–0,898)	0.04*
IL-1b	–0.92±0.24	–0.57±0.13	0.22	–.0±0.24	–0.54±0.13	0.10	–0.98±0.22	–0.82±0.12	0.0078**	0 (–0,638)	0.016*
GM-CSF	0.75±0.17	1.17±0.09	0.034*	0.74±0.16	1.17±0.009	0.030*	1.36±0.16	0.98±0.09	0.062	0.219 (0.054–0.973)	0.046*
IFN-γ	1.53±0.18	1.66±0.10	0.73	1.49±0.18	1.69±0.10	0.35	1.98±0.17	1.54±0.09	0.03*	0.036 (0.002–0.455)	0.0014**
MIP-1b	1.41±0.14	1.72±0.08	0.081	1.39±0.14	1.73±0.08	0.055	1.84±0.15	1.59±0.08	0.16	0.282 (0.113–0.875)	0.031*
MMP-9	4.89±0.15	5.50±0.08	0.0023**	4.98±0.16	5.54±0.09	0.016*	5.61±0.17	5.28±0.09	0.11	0.317 (0.109–1.099)	0.068

Abbreviations:
GSH: glutathione, GSSG: oxidized glutathione, Mo: monocyte, Mϕ: macrophage, R-Mo: reductive monocyte, O-Mo: oxidative monocyte, TIL: tumor infiltrating T lymphocyte, BRM: biological response modifier, tGSH: intracellular glutathione APC: antigen presenting cell, CTL: cytotoxic T lymphocyte, LNT: Lentinan, MMP: matrix metalloproteinase, MCB: monochlorobimane, DC: dendritic cell, PGE2: prostaglandin E2

immune cells are then infiltrated into tumor tissue and, as a result, this chain reaction induces and keeps specific Th1 immune responses working efficiently. As GM-CSF and IL-1ϕ, are known ?to induce Th1 responses, it is believed that these cytokines/chemokines will create a suitable inflammatory environment to boost antitumor immunity. IFN-γ is a typical Th1 cytokine and its presence reflects the occurrence of antitumor immune response. In this context, it is noteworthy that IFN-γ is a potent inducer of Mϕ transition to R-Mϕ, while Th2 cytokine IL-4 induces a transition to O-Mϕ.[15] It is interesting to note that GM-CSF tends to induce R-Mϕ, while M-CSF induces O-Mϕ (Hamuro, unpublished data).

Although further consideration is required of the plasma biomarkers for various tumors presented in this review before they can be used clinically, published results indicate that several cytokine/chemokine /MMP from frozen plasma samples can reliably be used as alternative biomarkers.

CONCLUSION

In this review, we discussed the notion that patients with poor antitumor immune responses have lower survival rates, and have introduced a method of ascertaining antitumor immune status using peripheral blood specimens before surgery. Predictions using peripheral blood markers will be helpful in determining therapeutic options for patients with solid tumors and more importantly allow for the optimal administration of presurgery patient-oriented treatments. These predictions are based on the idea that GSH status in Mo affect the clinical outcome of solid tumor bearing patients, and that knowing Mo status using easily obtainable plasma samples, will improve the prognosis of patients who may require treatment to boost their immune response prior to surgery. To induce this immune response we believe that BRM such as LTN and OK-432 are suitable options.

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REFERENCES

1. Naito Y, Saito K, Shiiba K, et al. CD8$^+$ T cells infiltrated within cancer cell nests as a prognostic factor in human colorectal cancer. *Cancer Res.* 1998;58:3491–3494.
2. Galon J, Costes A, Sanchez-Cabo F, et al. Type, density, and location of immune cells within human colorectal tumors predict clinical outcome. *Science.* 2006;313:1960–1964.
3. Galon J, Fridman WH, Pages F. The adaptive immunologic microenvironment in colorectal cancer: a novel perspective. *Cancer Res.* 2007;67:1883–1886.
4. Hamuro J, Takatsuki F, Suga T, Kikuchi T, Suzuki M. Synergistic antimetastatic effects of lentinan and interleukin 2 with pre- and postoperative treatments. *Jpn J Cancer Res.* 1994;85:1288–1297.
5. Oba K, Teramukai S, Kobayashi M, et al. Efficacy of adjuvant immunochemotherapy with polysaccharide K for patients with curative resections of gastric cancer. *Cancer Immunol Immunother.* 2007;56:905–911.
6. Kojima S, Nakayama K, Ishida H. Low dose gamma-rays activate immune functions via induction of glutathione and delay tumor growth. *J Radiat Res (Tokyo).* 2004;45:33–39.
7. Fujiwara Y, Kamikonya N, Inoue T, et al. Chemoradiotherapy for T3 and T4 squamous cell carcinoma of the esophagus using low-dose FP and radiation: a preliminary report. *Oncol Rep.* 2005;14:1177–1182.

8. Uno K, Okuno K, Kato T, *et al.* Pre-operative intracellular glutathione levels of peripheral monocytes as a biomarker to predict survival of colorectal cancer patients. Can Imm Imm. 2010;59:1457–1465.
9. Uekusa Y, Ogawa M, Gao P, *et al.* The development of peritumoral stroma required for IL-12 induced tumor regression depends on the T cell/IFN-gamma-involving host-tumor interaction. Int J Oncol. 2000;16:805–814.
10. Matsuzaki T, Okuno K, Uno K, *et al.* Intracellular glutathione (GSH) of monocytes (Mo) directs the development of peritumoral stroma in patients with colon carcinoma [in Japanese]. Gan To Kagaku Ryoho. 2005;32:1537–1539.
11. Hamuro J, Murata Y, Suzuki M. The triggering and healing of tumor stromal inflammatory reactions regulated by oxidative and reductive macrophages. Gann Monogr Cancer Res. 1999;48:153.
12. Peterson JD, Herzenberg LA, Vasquez K, Waltenbaugh C. Glutathione levels in antigen-presenting cells modulate Th1 versus Th2 response patterns. Proc Natl Acad Sci U S A. 1998;95:3071–3076.
13. Mills CD, Kincaid K, Alt JM, Heilman MJ, Hill AM. M-1/M-2 macrophages and the Th1/Th2 paradigm. J Immunol. 2000;164:6166–6173.
14. Mantovani A, Sozzani S, Locati M, Allavena P, Sica A. Macrophage polarization: tumor-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes. Trends Immunol. 2002;23:549–555.
15. Murata Y, Shimamura T, Hamuro J. The polarization of T(h)1/T(h)2 balance is dependent on the intracellular thiol redox status of macrophages due to the distinctive cytokine production. Int Immunol. 2002;14:201–212.
16. Murata Y, Ohteki T, Koyasu S, Hamuro J. IFN-gamma and pro-inflammatory cytokine production by antigen-presenting cells is dictated by intracellular thiol redox status regulated by oxygen tension. Eur J Immunol. 2002;32:2866–2873.
17. Utsugi M, Dobashi K, Ishizuka T, *et al.* c-Jun N-terminal kinase negatively regulates lipopolysaccharide-induced IL-12 production in human macrophages: role of mitogen-activated protein kinase in glutathione redox regulation of IL-12 production. J Immunol. 2003;171:628–635.
18. Utsugi M, Dobashi K, Koga Y, *et al.* Glutathione redox regulates lipopolysaccharide-induced IL-12 production through p38 mitogen-activated protein kinase activation in human monocytes: role of glutathione redox in IFN-gamma priming of IL-12 production. J Leukoc Biol. 2002;71:339–347.
19. Alam K, Ghousunnissa S, Nair S, Valluri VL, Mukhopadhyay S. Glutathione-redox balance regulates c-rel-driven IL-12 production in macrophages: possible implications in antituberculosis immunotherapy. J Immunol. 2010;184:2918–2929.
20. Short S, Merkel BJ, Caffrey R, McCoy KL. Defective antigen processing correlates with a low level of intracellular glutathione. Eur J Immunol. 1996;26:3015–3020.
21. Lemaire G, Guittet O, Vesin MF, Lepoivre M, Cottet MH. Glutathione depletion reveals impairment of antigen processing and inhibition of cathepsin activity by nitric oxide in antigen-presenting cells. Mol Immunol. 2009;46:1100–1108.
22. Ma J, Liu L, Che G, *et al.* The M1 form of tumor-associated macrophages in non-small cell lung cancer is positively associated with survival time. BMC Cancer. 2010;10:112.
23. Sarkar K, Bose A, Chakraborty K, *et al.* Neem leaf glycoprotein helps to generate carcinoembryonic antigen specific anti-tumor immune responses utilizing macrophage-mediated antigen presentation. Vaccine. 2008;26:4352–4362.
24. Kuppner MC, Scharner A, Milani V, *et al.* Ifosfamide impairs the allostimulatory capacity of human dendritic cells by intracellular glutathione depletion. Blood. 2003;102:3668–3674.
25. Liang CM, Lee N, Cattell D, Liang SM. Glutathione regulates interleukin-2 activity on cytotoxic T-cells. J Biol Chem. 1989;264:13519–13523.
26. Desai A, Huang X, Warren JS. Intracellular glutathione redox status modulates MCP-1 expression in pulmonary granulomatous vasculitis. Lab Invest. 1999;79:837–847.
27. Hashimoto S, Gon Y, Matsumoto K, *et al.* Intracellular glutathione regulates tumour necrosis factor-alpha-induced p38 MAP kinase activation and RANTES production by human bronchial epithelial cells. Clin Exp Allergy. 2001;31:144–151.
28. Zhao W, Darmanin S, Fu Q, *et al.* Hypoxia suppresses the production of matrix metalloproteinases and the migration of human monocyte-derived dendritic cells. Eur J Immunol. 2005;35:3468–3477.
29. Ohno N, Egawa Y, Hashimoto T, Adachi Y, Yadomae T. Effect of beta-glucans on the nitric oxide synthesis by peritoneal macrophage in mice. Biol Pharm Bull. 1996;19:608–612.
30. Chihara G, Hamuro J, Maeda YY, *et al.* Antitumor and metastasis-inhibitory activities of lentinan as an immunomodulator: an overview. Cancer Detect Prev Suppl. Suppl. 1987;1):423–443.
31. Okamoto H, Shoin S, Koshimura S, Shimizu R. Studies on the anticancer and streptolysin S-forming abilities of hemolytic streptococci. Jpn J Microbiol. 1967;11:323–326.
32. Okamoto M, Ohe G, Oshikawa T, *et al.* Enhancement of anti-cancer immunity by a lipoteichoic-acid-related molecule isolated from a penicillin-killed group A streptococcus. Cancer Immunol Immunother. 2001;50:408–416.
33. Uno K, Mitsuishi Y, Tanigawa M, *et al.* A series of immune responses leading to the induction of T cell IL-12/IL-18 responsiveness in patients with relatively large tumor burdens. Cancer Immunol Immunother. 2003;52:33–40.
34. Miyata T, Yamamoto S, Sakamoto K, Morishita R, Kaneda Y. Novel immunotherapy for peritoneal dissemination of murine colon cancer with macrophage inflammatory protein-1beta mediated by a tumor-specific vector, HVJ cationic liposomes. Cancer Gene Ther. 2001;8:852–860.
35. Luo X, Yu Y, Liang A, *et al.* Intratumoral expression of MIP-1beta induces antitumor responses in a pre-established tumor model through chemoattracting T cells and NK cells. Cell Mol Immunol. 2004;1:199–204.
36. Apte RN, Voronov E. Is interleukin-1 a good or bad "guy" in tumor immunobiology and immunotherapy? Immunol Rev. 2008;222:222–241.
37. McCune CS, Marquis DM. Interleukin 1 as an adjuvant for active specific immunotherapy in a murine tumor model. Cancer Res. 1990;50:1212–1215.
38. Sillanpaa S, Anttila M, Voutilainen K, *et al.* Prognostic significance of matrix metalloproteinase-9 (MMP-9) in epithelial ovarian cancer. Gynecol Oncol. 2007;104:296–303.