

by U5A2-13 mAb. A cDNA library was generated from murine BCL1 cells that were positive for U5A2-13 mAb using a pME18S expression vector. Not only NKT cells, but also some murine leukemia cell lines including BCL1, react with U5A2-13 mAb, but BCL1 expresses the most antigen (data not shown). A transfectant of a 2.8 kb cDNA clone, termed BLV-13, that was selected after two cycles of enrichment bound U5A2-13 mAb but not isotype-matched control IgG (Fig. 1A). Before sequencing this cDNA clone, we confirmed its enrichment in the second-round cDNA library by Southern blotting. Sequence analysis demonstrated that BLV-13 cDNA consisted of 2574 bp, encoding a polypeptide with 537 amino acid residues. A GenBank search revealed that the deduced amino acid sequence of BLV-13 was identical to mouse ICAM-1 (GenBank accession no. P13597).

We then Western blotted and immunoprecipitated lysates from BLV-13-transfected COS-7 cells using U5A2-13 and anti-ICAM-1 Abs. A band stained by anti-ICAM-1 Ab in the lysates of BLV-13-transfected COS-7 cells was dimin-

ished by its blocking peptides (Fig. 1B). Immunoprecipitates obtained from BLV-13-transfected COS-7 cells using U5A2-13 and anti-ICAM-1 Abs were detected as a 100 kDa protein band that corresponded to the molecular mass of mouse ICAM-1 (Fig. 1C). To confirm that U5A2-13 mAb reacts with mouse ICAM-1, hepatic mononuclear cells isolated from C57BL/6 mice and ICAM-1 deficient mice were dual-stained with anti-CD3 and one of U5A2-13, anti-ICAM-1 (clone 3E2) or anti-NK1.1 mAbs. We examined two strains of ICAM-1 mutant (exons 4 and 5) mice. U5A2-13 mAb was negative in both strains of ICAM-1 gene targeted mice (Fig. 2). These data demonstrated that our U5A2-13 mAb recognizes ICAM-1.

Furthermore, we sequenced 500 clones obtained from the cDNA library constructed after two cycles of enrichment (data not shown). One-hundred-and-twenty-three out of 500 clones had precisely the same cDNA insert as the 2.8 kb BLV-13 clone, suggesting the absence of an alternative splicing variant other than ICAM-1 mRNA encoding a specific protein recognized by U5A2-13 mAb. No other

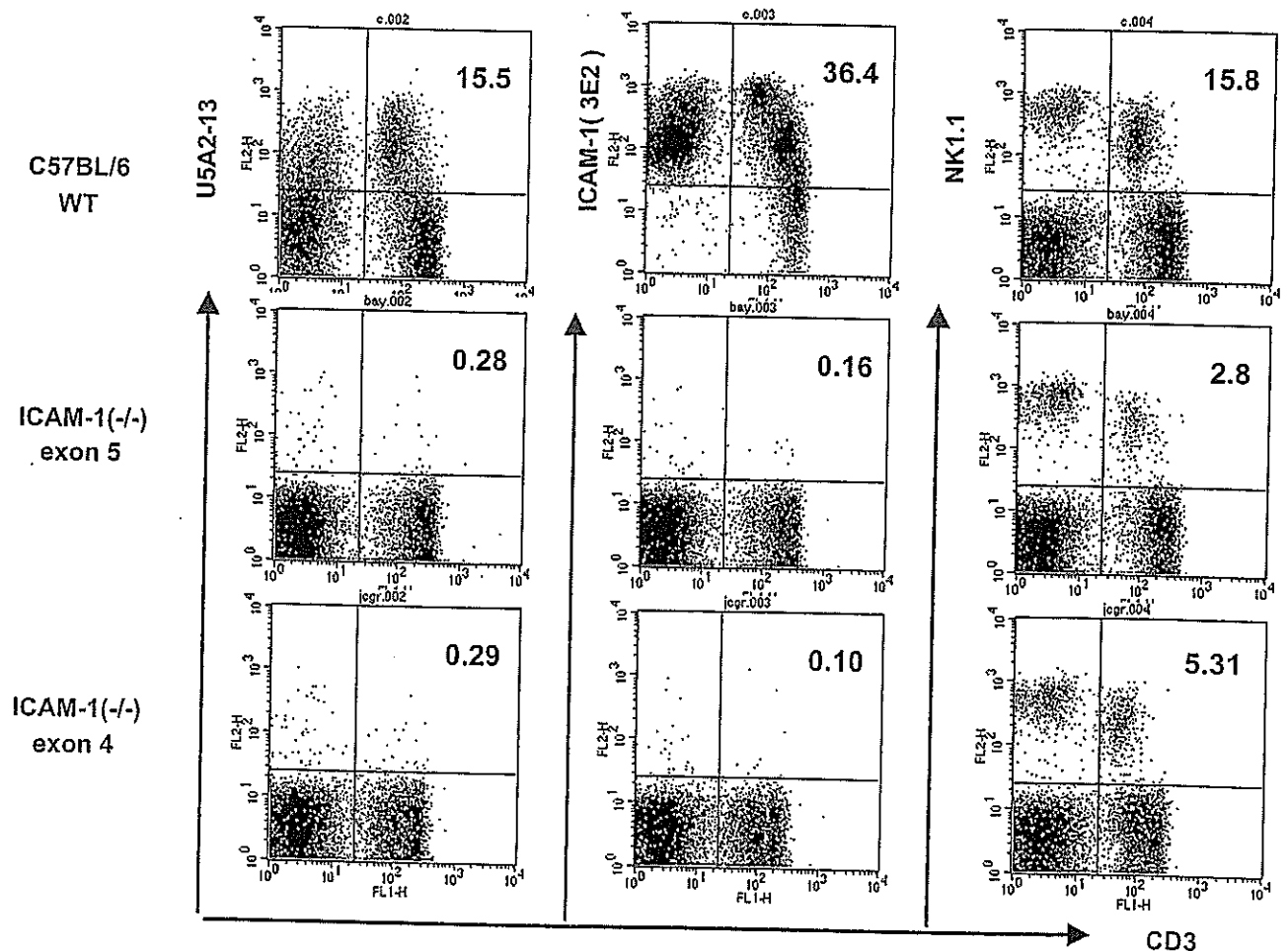


Fig. 2. U5A2-13 mAb did not react with lymphocytes in ICAM-1 deficient mice. Fresh hepatic mononuclear cells were isolated from C57BL/6 and two strains of mice deficient in ICAM-1 (exon 4 mutant and exon 5 mutant) then dual-stained with anti-CD3 mAb and one of U5A2-13 mAb, anti-ICAM-1 (clone 3E2) mAb, or anti-NK1.1 mAb. Data are representative of five individual experiments with similar results.

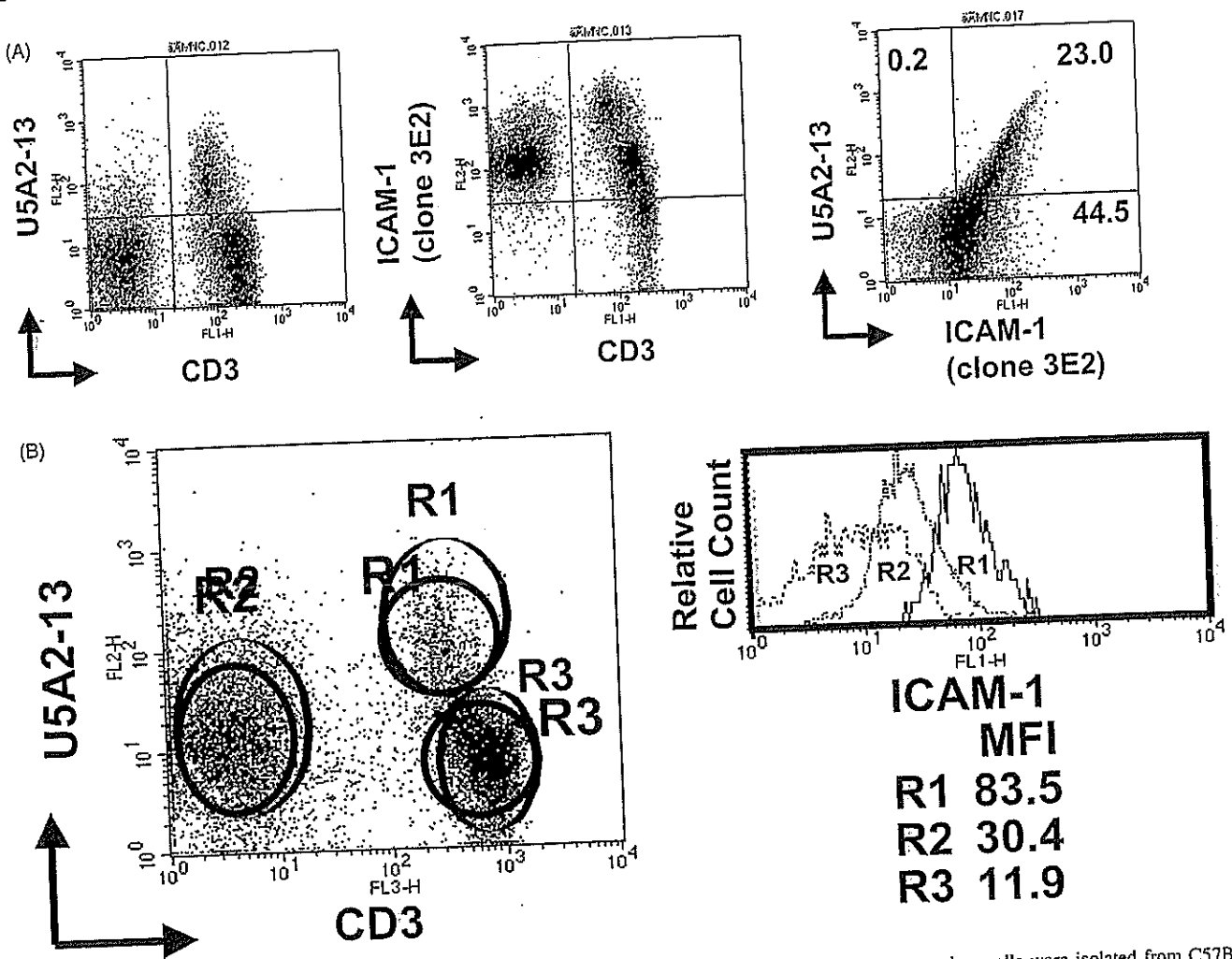


Fig. 3. Flow cytometry revealed that U5A2-13⁺ T cells correspond to ICAM-1^{high} T cells. Fresh hepatic mononuclear cells were isolated from C57BL/6 and stained with anti-CD3 mAb, U5A2-13 mAb and anti-ICAM-1 (clone 3E2) mAb (A). Mean fluorescence intensity (MFI) of ICAM-1 for each gated region is indicated below the panel (B). Data are representative of three individual experiments with similar results.

genes encoding a cell surface protein were found among the remaining 377 clones.

3.2. NKT cells express an epitope recognized by U5A2-13 mAb in extracellular domain two of ICAM-1

The profiles of hepatic mononuclear cells dual-stained with anti-CD3 mAb and one of U5A2-13, or anti-ICAM-1 (clone 3E2) mAbs were quite different (Fig. 3A). Three-color flow cytometry demonstrated that the U5A2-13 positive population encompasses CD3 positive, ICAM-1 (3E2) bright subsets, corresponding to approximately 35% of the 3E2 positive population (Fig. 3B). Flow cytometry revealed that the mean fluorescence intensity of ICAM-1 is very high not only murine, but also in human TCR V α 24 positive NKT cells (unpublished data).

We examined whether or not the epitopes of the antibodies are different, by performing a competitive inhibition assay. U5A2-13 mAb staining was not blocked by a prior

incubation with other anti-ICAM-1 mAbs such as clone 3E2, KAT-1, or YN1/1 (data not shown).

In addition, in order to determine U5A2-13 epitope, we generated synthetic peptides that represent amino acid sequences of ICAM-1 and examined their reactivity with U5A2-13 mAb by ELISA. Synthetic rod-attached peptides were used to map the antigenic sites on the extracellular domain of the ICAM-1. By means of the Pepscan method, all possible 2230 overlapping 30-mer peptides, composed of two 15-mer parts derived from the primary sequence, were synthesized. U5A2-13 mAb was tested against the synthetic minicard-peptides. Peptides were considered to represent antigenic sites if peaks occurred in a set of neighboring peptides and if at least one of the peaks in such a set amounted to more than three times the background. U5A2-13 mAb recognized a discontinuous epitope that was made of three parts of extracellular domain two of ICAM-1. The identified sites are: 161–178 region in B–C loop, 135–151 region in C'–E loop, and 188–204 region in F–G loop (Fig. 4).

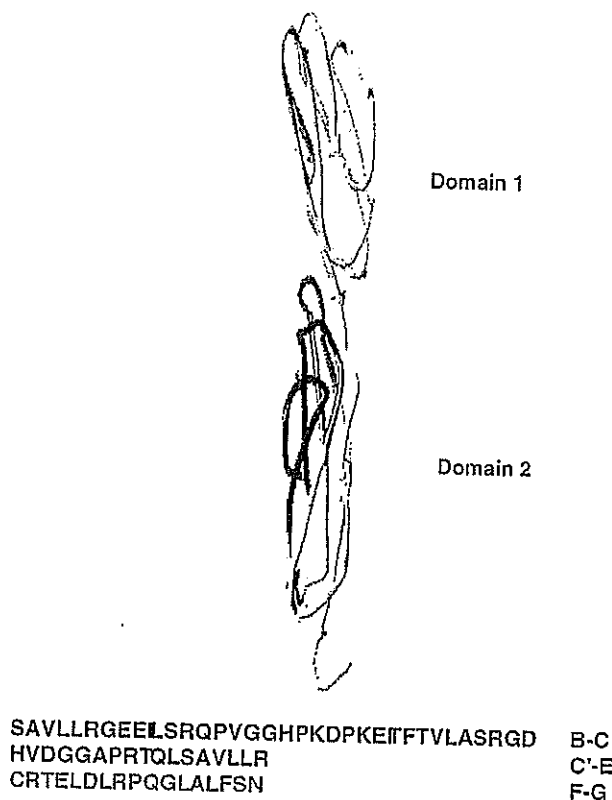


Fig. 4. U5A2-13 epitope in a 3D model of human ICAM-1. U5A2-13 epitope consists of three loops in domain two of ICAM-1. The three homologous regions are demonstrated in a model of human ICAM-1 (GenBank accession no. MMD8 8008). The core epitope is depicted in red. Two adjacent loops in blue and green are also part of the epitope.

Collectively, these data suggest that NKT cells express a unique epitope of ICAM-1 recognized by U5A2-13 mAb.

4. Discussion

Our studies demonstrated that an NKT cell surface antigen recognized by a novel mAb, U5A2-13, is encoded by *ICAM-1* gene and that its discontinuous epitope is composed of three loops located in extracellular domain two of ICAM-1. Expression of the classical NK marker, NK1.1 antigen, is confined to C57BL/6 and related strains, which hampers investigation in disease models of NK1.1-negative strains [1,2]. We showed previously that U5A2-13⁺ T cells encompass a population functionally similar to NK1.1⁺ T cells in various mouse strains such as C57BL/6, BALB/c, and C3H/He [28,30,31]. Furthermore, staining with U5A2-13 mAb yielded higher signal intensity for NKT cells than NK cells in FACS[®] analyses. This is unique compared with antibodies of other NK markers such as NK1.1, IL-2R β , Ly49 or DX5.

Recently the antigen recognized by DX5 has been molecularly cloned, which revealed CD49b (Very Late Antigen-2) [41]. Like U5A2-13, a surrogate NK marker, DX5, also corresponds to an adhesion molecule. This is not fortuitous but rather suggests that the importance of adhesion molecules

cannot be over-emphasized in NK and NKT cell function. NKT cells are considered to activate NK cells rapidly in the innate immune system [9]. NK cells express large amount of LFA-1 [42]. Our study showed that NKT cells express ICAM-1 most. This constitutes a reasonable evidence for efficient cross-talk between these cells.

The staining profiles of hepatic MNCs with U5A2-13 mAb and with other conventional anti-ICAM-1 mAbs (such as 3E2, YN1/1 or KAT-1) were quite different (Fig. 3A). U5A2-13 epitope resides in domain two. The epitopes of the other anti-ICAM-1 antibodies, on the other hand, are located in domain one, which is the site responsible for binding with its ligand LFA-1. Therefore, in competitive inhibition assay, staining with U5A2-13 mAb was not blocked by these antibodies (unpublished observation). We cannot exclude a possibility that, because of its affinity for ICAM-1, U5A2-13 mAb recognizes only the ICAM-1^{high} population when ICAM-1 is present in substantial amounts on the cell surface. Another possibility is that freshly isolated naïve NKT cells express a unique epitope in domain two of ICAM-1 in such a manner that it can efficiently react with U5A2-13 mAb. The ICAM-1 epitope recognized by U5A2-13 mAb may depend on glycosylation selective to NK cells. Accumulating evidence shows that ICAM-1 forms homodimers on the cell surface that are more efficient than monomers in binding to its ligand, LFA-1 [43–46]. In freshly harvested mononuclear cells, staining with U5A2-13 mAb is mostly confined to NKT cells but not to conventional T and B cells. We also revealed that, upon activation by a mitogen, not only NKT cells but also T and B cells start to react with U5A2-13 mAb [47]. Taken together, these findings may indicate that homodimers of ICAM-1 on the surface of activated ICAM-1^{high} cells form an epitope for U5A2-13 mAb. ICAM-1 per se is widely expressed on lymphocytes but it is not until they get fully activated and become ICAM-1^{high} when they begin to show U5A2-13 epitope otherwise concealed. Similarly, NK1.1 antigen is also induced on CD8⁺ T cells upon activation [48].

It is of interest that this U5A2-13 epitope on NKT cells is related to their recognition and functions. In vivo administration of U5A2-13 mAb not only modulates cytokines production by NKT cells upon stimulation with α -GalCer but also increases tumor metastases in a B16 melanoma model (manuscript in preparation). We conclude that NKT cells express a unique U5A2-13 epitope of ICAM-1.

Acknowledgements

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ROLE OF NATURAL KILLER T CELLS IN ANTI-TUMOR IMMUNE RESPONSE

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1. Introduction

Natural killer T (NKT) cells are reportedly involved in tumor rejection. NKT cells in mice are usually defined as lymphocytes expressing intermediate levels of TCR and NK cell-associated molecules, particularly NK1.1. They predominantly express TCR with invariant V α 14J α 281/V β 8, 7, or 2 in mice or V α 24J α Q/V β 11 in humans, and most are phenotypically double-negative (CD4⁻CD8⁻) or single-positive (CD4⁺CD8⁻) T cells. These cells produce large amounts of interferon (IFN)- γ and interleukin (IL)-4, suggesting that they play an important role in regulating Th1/Th2 balance, including tumor immunity. Most NKT cells are restricted by CD1d molecule and react strongly to sponge-derived glycolipid α -galactosylceramide (α -GalCer)¹.

2. NKT cells express a unique epitope of ICAM-1 that plays a role in tumor rejection

2.1 Establishment of monoclonal antibody U5A2-13: NKT cells in mice are generally defined as NK1.1⁺ T cells, although NK1.1 antigen is expressed only in C57BL/6 and related strains. This has precluded investigations of other strains. To identify a novel NKT cell-surface marker irrespective of strain, we generated the monoclonal antibody (mAb) U5A2-13², which phenotypically and functionally identifies populations that are similar to NKT cells. This mAb was originally obtained by immunizing a Fischer rat with tMK-2U lymphoma cells from a BALB/c nude mouse carrying xenografted human inflammatory breast tumor cells.

We have shown that U5A2-13⁺ T cells predominantly use TCR V β 8, 7 or 2, and that,

similar to NK1.1⁺ T cells, U5A2-13⁺ T cells can produce both IFN- γ and IL-4 upon cross linking with CD3³). We have also demonstrated that hepatic U5A2-13⁺ T cells recognize the NKT cell ligand, α -GalCer, as presented by CD1d molecules on dendritic cells⁴). These results indicate that U5A2-13 mAb represents a valuable tool for studying NKT cells in NK1.1-negative mouse strains. However, flow cytometry revealed that the cell surface molecule recognized by U5A2-13 mAb differs from other NK lineage molecules such as NK1.1, Ly-49, 2B4 and DX5⁵). To elucidate the U5A2-13 antigen on NKT cells, the antigen was cloned using expressional cloning. The amino acid sequence of BLV-13 clone was identical to mouse ICAM-1. To confirm that U5A2 reacts with ICAM-1, hepatic mononuclear cells from B6 and ICAM-1 mutant mice were stained. U5A2 was negative in two available strains of ICAM-1 K/O mice. These data demonstrated that our U5A2 recognizes really ICAM-1. Note that the staining pattern of U5A2-13 mAb differs considerably from that of ICAM-1. U5A2-13 mAb was found to recognize ICAM-1 high-expressing cell population and this population is around 30% of ICAM-1-positive T cells. Our results seem to indicate that U5A2-13 mAb recognizes a unique epitope expressed only in NKT cells. We therefore performed epitope mapping and revealed that the U5A2 epitope comprises three loops of the extracellular domain 2 of ICAM-1, whereas all available Abs recognize epitopes of domain 1. Prior studies have shown that the number of NKT cells is decreased in LFA-1 K/O mice. In our study, the number of NKT cells was found to be further decreased in ICAM-1-deficient mice. This suggests that ICAM-1/LFA-1 interactions and associated signaling pathways are important in the differentiation of NKT cells.

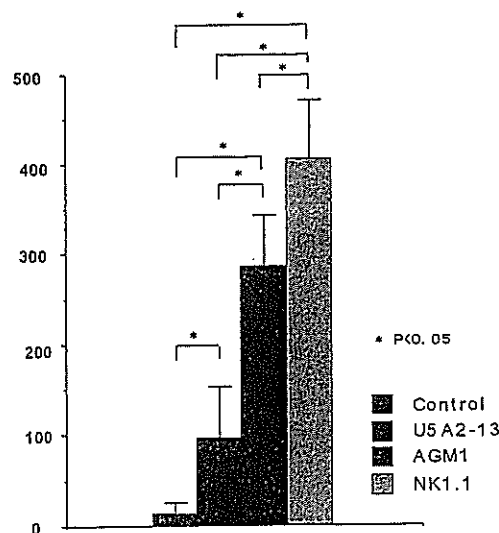


Fig.1 B16 Melanoma Lung Metastases after Ab Treatment

2.2 A unique epitope of ICAM-1 that plays a role in tumor rejection: Here, we investigated whether the U5A2-13 epitope on NKT cells is responsible for anti-tumor activities. To assess the contribution of NKT cells compared with NK cells in a lung metastasis model, mice were treated with U5A2-13 mAb, anti-asialo GM1 (AGM1) Ab, and anti-NK1.1 mAb, then inoculated with B16 melanoma cells. In contrast with anti-NK1.1 antibody, *in vivo* treatment with U5A2-13 did not reduce the number of NKT cells, but resulted in shedding of ICAM-1 from the NKT cell surface, thus increasing serum levels of soluble ICAM-1. U5A2-13 mAb *in vivo* down-regulated U5A2-13 epitope expression, whereas numbers of NK1.1⁺ T cells remained stable. Anti-AGM1 Ab *in vivo* depleted NK cells, whereas numbers of NK1.1⁺ and U5A2-13⁺ T cells remained stable. Anti-NK1.1 mAb *in vivo* depleted NK, NK1.1⁺ T and U5A2-13⁺ T cells. Tumor growth-promoting effects were augmented by, in ascending order, U5A2-13 mAb, anti-AGM1 Ab, and anti-NK1.1 mAb (Fig.1). Collectively, these data suggest that the presence of U5A2-13 epitope is important for NKT cells to control tumor metastasis in the B16 melanoma model.

3. Expansion of Th0-type NK1.1⁻ CD1d-restricted NKT cells by α -GalCer stimulation

3.1 Expansion of three distinct NKT cell populations by IL-2 and α -GalCer: To characterize α -GalCer-activated NKT cells *in vitro*, murine splenic mononuclear cells (MNCs) were cultured with or without 50 ng/ml of α -GalCer and 100 U/ml of human IL-2. Before culturing, a small percentage of NK1.1⁺ T cells (1.01±0.24%) and CD1d/ α -GalCer tetramer⁺ T cells (1.01±0.18%) were identified in splenic MNCs. Percentages of NK1.1⁺CD1d/ α -GalCer tetramer⁺ cells and NK1.1⁻CD1d/ α -GalCer tetramer⁺ cells in T cells were 0.95±0.52% and 0.88±0.54%, respectively. On day 6, percentages of NK1.1⁺ T cells and NK cells were increased when cultured with IL-2 and α -GalCer or IL-2 alone. However, the percentage of CD1d/ α -GalCer tetramer⁺ T cells was increased in culture with IL-2 and α -GalCer, but not in culture with IL-2 alone. These results indicate that IL-2 induces expansion of NK1.1⁺ T cells, whereas α -GalCer induces expansion of CD1d/ α -GalCer tetramer⁺ T cells that are able to react to α -GalCer.

Next, we examined whether CD1d/ α -GalCer tetramer⁺ NKT cells express NK1.1 molecules. Almost none of the CD1d/ α -GalCer tetramer⁺ NKT cells expanded with IL-2 and α -GalCer expressed NK1.1 molecules. This indicates that three distinct murine NKT-cell subsets are expanded after IL-2 and α -GalCer activation *in vitro*: NK1.1⁻CD1d/ α -GalCer tetramer⁺ NKT cells; NK1.1⁺CD1d/ α -GalCer tetramer⁺ NKT cells; and NK1.1⁺CD1d/ α -GalCer tetramer⁻ NKT cells. In freshly isolated splenic MNCs, NK1.1⁻CD1d/ α -GalCer tetramer⁺ NKT cells and NK1.1⁺CD1d/ α -GalCer tetramer⁺ NKT cells comprised CD4⁺CD8⁻ cells (70-80%) and CD4⁻CD8⁻ (DN) cells (20-30%). α -GalCer-activated NK1.1⁻ CD1d/ α -

GalCer tetramer⁺ NKT cells also consist of CD4⁺CD8⁻ T cells (50±8%) and CD4⁻CD8⁻ (DN) T cells (47±5%). In contrast to NK1.1⁻CD1d/α-GalCer tetramer⁺ NKT cells, NK1.1⁺CD1d/α-GalCer tetramer⁻ NKT cells (with or without α-GalCer) comprised CD4⁻CD8⁺ cells and DN cells. Approximately half of the α-GalCer-activated CD8⁺NK1.1⁺ cells were CD8 αα homodimers. NK1.1⁺CD1d/α-GalCer tetramer⁺ NKT cells expanded with IL-2 and α-GalCer consisted of CD4⁺ cells, DN cells and approximately 10% CD8⁺ cells.

3.2 Cytokine production by three subsets of *ex vivo* α-GalCer activated-NKT cells: Several investigators have reported that NKT cells secrete large amounts of cytokines after α-GalCer or TCR/CD3 stimulation, particularly IFN-γ and IL-4. We therefore examined whether α-GalCer activated-NKT cells produce these cytokines. After culture with IL-2 and α-GalCer or IL-2 alone, supernatants were harvested on days 4, 6 and 8 and analyzed for IFN-γ and IL-4 production by ELISA. Splenic MNCs cultured with IL-2 and α-GalCer produced large amounts of IL-4 (16.9±2.2 ng/ml) and IFN-γ (22.0±3.4 ng/ml) from day 4 to day 6. However, these cytokines could not be detected in supernatant from cells cultured with IL-2 alone.

Next, we examined which NKT cell subsets produce IFN-γ or IL-4. On day 6, cultured cells were collected and stained with intracellular IFN-γ or IL-4. Remarkably, a clear difference in cytokines produced by NK1.1⁻CD1d/α-GalCer tetramer⁺ NKT cells, NK1.1⁺CD1d/α-GalCer tetramer⁻ NKT cells and NK1.1⁺CD1d/α-GalCer tetramer⁺ subsets were observed. NK1.1⁻CD1d/α-GalCer tetramer⁺ NKT cells produced IFN-γ and IL-4, whereas NK1.1⁺CD1d/α-GalCer tetramer⁻ NKT cells and NK1.1⁺CD1d/α-GalCer tetramer⁺ NKT cells only produced IFN-γ. In contrast to α-GalCer-activated NKT cells, intracellular cytokines could not be detected with cells cultured using IL-2 alone. In NK1.1⁻CD1d/α-GalCer tetramer⁺ NKT cells, both CD4⁺ and DN T cells produced IFN-γ and/or IL-4. In NK1.1⁺CD1d/α-GalCer tetramer⁻ NKT cells, both CD8⁺ cells and DN T cells produced IFN-γ.

4. Expansion of α-GalCer-Stimulated human V_α24⁺ NKT Cells under non-FBS Culture Conditions

Efficient expansion of V_α24⁺ NKT (V_α24⁺ CD3⁺) cells from granulocyte colony-stimulating factor (G-CSF)-mobilized peripheral blood (PB) cells is possible under culture conditions with α-GalCer, IL-2 and fetal bovine serum (FBS) *in vitro*⁶. In an attempt to avoid potential risks associated with FBS and to establish a clinical-scale expansion system, we investigated the development of a more efficient non-FBS expansion system for V_α24⁺ NKT cells with α-GalCer and IL-2.

4.1 Efficient expansion of V α 24⁺ NKT cells in autologous plasma: In order to investigate a suitable non-FBS medium for V α 24⁺ NKT cell expansion, PB mononuclear cells (PBMCs) obtained from PB of normal healthy volunteers were cultured in medium containing α -GalCer, IL-2 and conditioned medium comprising: 10% FBS; 10% recombinant human serum albumin (rHSA); and 10% or 5% autologous plasma or 10% or 5% autologous serum. V α 24⁺ NKT cells were unable to proliferate in medium containing rHSA. Medium containing 5% autologous plasma promoted optimal growth of V α 24⁺ NKT cells both in the presence of α -GalCer and IL-2. No significant differences were observed between V α 24⁺ cell expansion in 10% or 5% autologous plasma or serum.

4.2 G-CSF mobilization augmented V α 24⁺ NKT cell expansion: Next, we studied culture efficiency for PBMCs and medium containing 5% autologous plasma derived from G-CSF-mobilized donors. Expansion ratios of V α 24⁺ NKT cells in a combined culture of PBMC and plasma before and after G-CSF mobilization were approximately 350-fold (before) and 2000-fold (after), respectively. Both PBMCs and plasma obtained from PB of normal healthy donors after G-CSF mobilization displayed maximal conditions for expansion of V α 24⁺ NKT cells. Although the percentage of V α 24⁺ NKT cells in G-CSF-mobilized PB was dramatically decreased compared to that from non-mobilized donors, V α 24⁺ NKT cells after G-CSF mobilization expanded more efficiently than before G-CSF mobilization. These results suggest that selective expansion of V α 24⁺ NKT cells is induced by administration of G-CSF.

4.3 Both G-CSF-mobilized PBMC and associated plasma affect V α 24⁺ NKT cell expansion: To determine which components affect cell expansion after G-CSF mobilization, several cross-combinations of pre- and post-G-CSF conditioned PBMCs and plasma were used. Optimal combination was obtained when post-G-CSF conditioned PBMCs and post-G-CSF conditioned plasma were combined. These results suggest that the G-CSF mobilization procedure conferred a proliferating advantage to V α 24⁺ NKT cells upon both PBMCs and associated plasma.

4.4 G-CSF induced changes in cytokine concentration in PB: Analysis of plasma before and after G-CSF mobilization revealed that levels of serum cytokines such as G-CSF, IL-3, IL-7 and IL-13 were increased after mobilization.

4.5 Kinetics of expanded cell populations with IL-2 and α -GalCer: Cell subsets in an expansion of V α 24⁺ NKT cells were monitored on days 0, 7, 14 of culture using apheresis of PBMCs in the presence of autologous plasma. Of note is the fact that on day 0, the percentage of V α 24⁺ NKT cells was extremely low (0.10%). The percentage of monocytes was approximately 30%, which was markedly higher than that observed before G-CSF administration (2.7-7.9%). The CD4/CD8.T cell ratio was >1. V α 24⁺ NKT cells under

stimulation with α -GalCer displayed linear propagation until day 14. CD8 T cells (but not CD4 T cells) also expanded, becoming the predominant population. The CD4/CD8 T cell ratio was thus reversed to <1 . Both B cells and monocytes completely disappeared by day 14 (2.33% and 0.16%, respectively). Given the results of our previous study, the abnormally extended percentage of monocytes on day 0 might contribute to an efficient expansion of $V_{\alpha}24^{+}$ NKT cells.

4.6 Intracellular cytokine production and cytotoxic activity of $V_{\alpha}24^{+}$ NKT cells: We purified expanded $V_{\alpha}24^{+}$ NKT cells after 14 days of culture and assessed both intracellular cytokine production and cytotoxic activity of these cells. The percentage of IFN- γ -producing cells among purified $V_{\alpha}24^{+}$ NKT cells was 75%, while that of IL-4-producing cells was 16%. Using PBMCs after G-CSF mobilization, $V_{\alpha}24^{+}$ NKT cells predominantly produced IFN- γ instead of IL-4. Cytotoxic assay was measured against several cell lines, including Daudi, K562, Jurkat, HCT-15, HT-29, WiDr, SKOV3, and OVCAR-3 lines. Expression of CD1d molecules on target tumor cells was evaluated using CD1d mAb. Some of these target cells (Daudi and Jurkat) expressed CD1d molecule, while others did not. Purified $V_{\alpha}24^{+}$ NKT cells displayed powerful cytotoxicity against all target tumor cell lines, particularly the hematological cell lines. This cytotoxicity was not related to expression of CD1d molecules on target cells. Expanded $V_{\alpha}24^{+}$ NKT cells in a combined culture of PBMCs and plasma obtained from apheresis after G-CSF mobilization retained their surface markers and production of IFN- γ and IL-4, and exerted powerful cytotoxic effects against tumor cells in a CD1d-independent manner. In conclusion, the results presented herein suggest that a new expansion strategy for $V_{\alpha}24^{+}$ NKT cells under non-FBS culture conditions using α -GalCer and IL-2 with G-CSF-mobilized PBMC and plasma offers the possibility of NKT immune cell therapy for cancer patients.

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Clinical Data

Clinicopathological statistics on registered prostate cancer patients in Japan: 2000 report from the Japanese Urological Association

CANCER REGISTRATION COMMITTEE OF THE JAPANESE UROLOGICAL ASSOCIATION

Abstract

Background: The purpose of the present paper was to investigate etiology, diagnosis, initial treatment, pathological findings and final outcomes for prostate cancer in Japan.

Methods: From 2001, the Japanese Urological Association initiated computer-based registration of prostate cancer patients in Japan to estimate etiology, diagnosis, initial planned treatment, pathological findings and final outcome.

Results: A total of 173 institutions responded and 4529 patients who were diagnosed with prostate cancer in 2000 were registered. In the first year, background factors, diagnostic procedures and initially planned treatment were recorded. The analysis of these registered cases is presented here. Nearly 30% of the cases demonstrated <10 ng/mL of prostate specific antigen. The six-core biopsy was the most commonly used procedure. The clinical T staging distribution was as follows: T1c, 20.3%; T2a, 21.8%; T2b, 17.3%; T3a, 15.8%; T3b, 11.0%; and T4, 8%. More than 70% of cases were diagnosed as M0. Hormone therapy alone was the initial treatment plan in more than half of the cases.

Conclusion: This is the first report on prostate cancer patients in Japan based on multi-institutional registration. Pathological findings and final outcome will be surveyed later by the Japanese Urological Association. After 5 years, adopted treatment, pathological results and final outcome will be registered.

Key words epidemiology, Japan, neoplasm, prostate.

Introduction

Prostate cancer is known around the world as a disease of elderly men. Parkin *et al.* reported that three-quarters of cases occur in men over 65 years of age.¹ In Japan, although the incidence of prostate cancer has been much less than in American and European countries, it has been sharply rising during the last two decades, a trend also observed in other Asian countries. Even before the introduction of prostate specific antigen (PSA) testing in the early 1990s, incidence rates had been rising in

many countries.^{1–5} Changes from traditional Japanese to more Western lifestyles and nutritional habits have been implicated in the increase in the incidence of prostate cancer in Japan.³ In the early 1990s, the PSA test contributed to the earlier diagnosis of prostate cancer, which is also reflected in the increased incidence. Now, in Japan as in other developed countries, prostate cancer is a large and growing public health problem whose risk factors are essentially unknown.

The precise nature of this problem differs from one country to another because of the wide range of national incidence and, to a much lesser degree, survival profiles that have been reported.^{1,5–8} Recently, however, Nakata *et al.*⁴ reported that the mortality rate of prostate cancer patients in Japan has been increasing as rapidly as the incidence, while in North America the mortality rate has actually declined even in the face of rising incidence.⁹

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The Japanese Urological Association (JUA) and the Japanese Society of Pathology revised the 2nd edition of *General Rule for Clinical and Pathological Studies on Prostate Cancer* to the 3rd edition in 2001¹⁰ and, in accordance with this new edition, initiated a study to estimate the etiology, diagnosis, initial treatment, pathological findings and final outcomes for prostate cancer in Japan by employing computer-based registration of prostate cancer patients from institutions all over Japan. The registration program has been established by the members of committee for revision of *General Rule for Clinical and Pathological Studies on Prostate Cancer*. Here we report the background data at diagnosis from the initial registration of 4529 patients who were diagnosed pathologically with prostate cancer in 2000.

Methods

Using a CD-ROM program developed by the members of committee for revision of *General Rule for Clinical and Pathological Studies on Prostate Cancer* and the staff of the National Cancer Center, new patients who were diagnosed by pathology to have prostate cancer in 2000 were registered. Age, occupation, race, concomitant malignancy, family history, past history and symptoms were entered as background factors for each prostate cancer patient. The findings from digital rectal examination (DRE), imaging, PSA level, number of cores taken by biopsy, number of positive core(s), histology of the biopsy specimen, Gleason score, 5th tumor, nodes, metastases (TNM) classification (5th edition), diagnostic process and initial treatment plan were also entered. The collected data were analyzed.

Results

One hundred and seventy-three institutions responded and 4529 patients who were diagnosed with prostate cancer in 2000 were registered. Approximately 14 000 new prostate cancer patients were estimated in Japan this year;⁹ therefore, approximately 30% of total cases were covered by this registry.

Background data

The age distribution of patients is summarized in Figure 1. More than one-quarter of all patients were 70–74 years old, and three-quarters were 60–79 years old. The ethnic distribution was as follows: Japanese, 99.56%; non-Japanese Mongolians, 0.29%; and Caucasian, 0.13%. Only 71 patients (1.57%) had a family

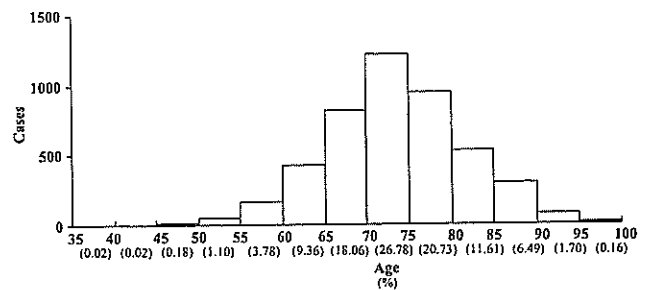


Fig. 1 Age distribution of prostate cancer newly diagnosed in 2000 and registered. Registered cases were divided into 5-year age groups as indicated. Bars indicate the number of cases equal to and above the age on the left lower corner and below the age on the right lower corner of each bar. The numbers below each bar indicate the percentage of cases in each age group among all registered cases.

Table 1 Findings of local imaging study

	<i>n</i>	(%)
Not visible	1255	27.71
Confirmed within prostate	1580	34.89
Extra-capsule	732	16.16
Invades adjacent structure	685	15.12
Uncertain	277	6.12
Total	4529	100.00

history of prostate cancer in either their father (0.66%), brother (0.82%), or uncle (0.09%). Common occupations of the registered patients were the following: retired (61.29%), office clerks or associate professionals (8.06%), corporate or general managers (6.93%), agricultural, forestry, or fishery workers (6.71%), and professionals or technicians (6.62%). A total of 520 patients (11.48%) had a history of other malignancies.

Initial evaluation

Of all the patients, 71.08% (3219 cases) presented with some symptoms and 339 cases (7.49% of all and 35.17% of metastatic disease) presented with symptoms from metastases.

The most commonly used staging modalities were transrectal ultrasonography (TRUS; 78.32%) and bone scan (73.53%), followed by computed tomography (CT; 66.75%) and magnetic resonance imaging (MRI; 42.77%). The findings from local imaging studies are summarized in Table 1. As expected, local imaging studies yielded better sensitivity (66.17% produced a positive finding) than DRE (58.29% true-positives; Table 2).

Table 2 Findings of digital rectal examination

	<i>n</i>	(%)
Not palpable	1706	37.67
One lobe	1090	24.07
Both lobe	760	16.78
Extra-capsule	481	10.62
Seminal vesicle invasion	113	2.50
Extra-prostate	196	4.33
Uncertain	18.3	4.04
Total	4529	100.00

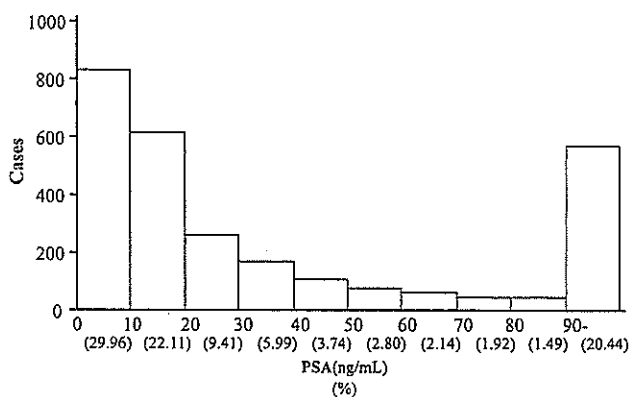


Fig. 2 Distribution of prostate specific antigen (PSA) value of registered cases. Registered cases were divided into groups of PSA value by 10 ng/mL as indicated. The PSA value was measured by Tandem-R and includes all Tandem-R compatible kits, such as *E-test* Tosoh, Chemilumi ACS-PSA, Shifalite PSA and Lumipulse PSA. Bars indicate the number of cases equal to and above the value on the left lower corner and below the value on the right lower corner of each bar. Cases with PSA \geq 90 ng/mL were counted as a single group. The numbers below each bar indicate the percentage of cases in each group among all registered cases.

The PSA level distribution is summarized in Figure 2. Nearly 30% of prostate cancer cases diagnosed in 2000 demonstrated a PSA value of <10 ng/mL.

A summary of the biopsy statistics is shown in Tables 3–5 and Figure 3. Because the same number of cores is usually taken from each lobe, the 5th, 7th, and 9th core samples are supposed to be additional targeted biopsies of suspicious lesions (Table 3). The percentage of biopsies yielding all positive cores was higher for the 6-core than for the 8-core biopsy (Fig. 3). However, the percentage of biopsies yielding only one positive core was higher for the 8-core procedure. A total of 99.8% of tumors were determined pathologically to be adenocarcinoma, followed by neuroendocrine tumors (0.1%). Moderately differentiated adenocarcinoma (43.5%) was

Table 3 Number of adopted biopsy cores

Core(s)	<i>n</i>	(%)
1	5	0.12
2	93	2.30
3	52	1.28
4	264	6.52
5	90	2.22
6	2128	52.52
7	282	6.96
8	616	15.20
9	90	2.22
≥ 10	432	10.66
Total	4052	100

Uncertain, 168 patients.

Table 4 Primary Gleason grade

Gleason grade	<i>n</i>	(%)
1	376	8.30
2	541	11.90
3	1155	25.50
4	695	15.30
5	308	6.80
Uncertain	1454	32.10
Total	4529	100

Table 5 Secondary Gleason grade

Gleason grade	<i>n</i>	(%)
1	251	5.50
2	552	12.20
3	1020	22.50
4	780	17.20
5	312	6.90
Uncertain	1614	35.60
Total	4529	100

the most common differentiation state found in this population, followed by well-differentiated (31.3%) and poorly differentiated adenocarcinomas (20.6%). Gleason grade classification is summarized in Tables 4,5; grade 3 was found to be the most common grade in both primary and secondary dominant tumors.

Initial staging

Clinical T stage of this population is summarized in Figure 4. The clinical T staging distribution is as follows: T1c, 20.3%; T2a, 21.8%; T2b, 17.3%; T3a, 15.8%; T3b, 11.0%; and T4, 8%. Regionally limited lymph node metastases examined mainly by CT were found in 501 cases (11.06%), and 3569 cases

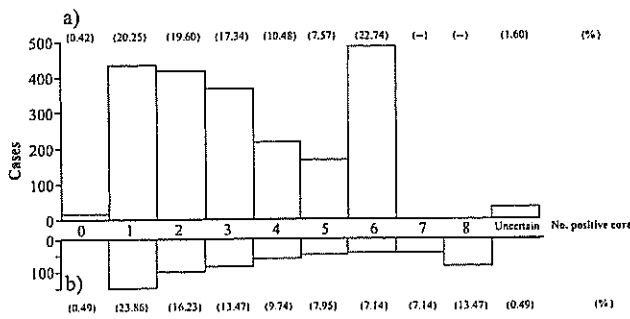


Fig. 3 Number of cancer-positive cores in (a) 6-core biopsy ($n = 2128$) and (b) 8-core biopsy ($n = 616$). Registered cases were divided into groups by number of cancer-positive cores as indicated. Bars indicate the number of cases in each group. The percentage of cases in each group among each subgroup of 6- or 8-core biopsy is indicated over or below the bars.

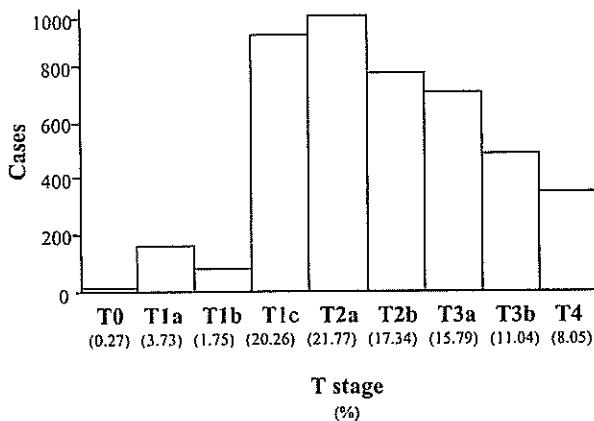


Fig. 4 Distribution of local staging (T staging). Registered cases were divided into groups by local staging as indicated according to the 1997 International Union Against Cancer (UICC) tumor, nodes, metastases (TNM) classification. Bars indicate the number of patients for each T stage. The numbers below each bar indicate the percentage of cases in each age group among all registered cases.

(78.80%) were diagnosed as negative lymph node metastasis.

Distant metastases are detailed in Table 6. More than 70% of patients were negative for distant metastasis.

Initial treatment strategies

Selected plans are summarized in Figure 5. Hormonal therapy alone was chosen for more than half of the cases. Prostatectomy with or without hormonal therapy was planned for 27.4% (1240 cases). Irradiation with or without hormonal therapy was not very common (8.1%). For cases of T1c to T3N0M0 (2671 cases total),

Table 6 M stage

M stage	<i>n</i>	(%)
M0	3243	71.61
M1a	40	0.88
M1b	861	19.01
M1c	63	1.39
MX	322	7.11
Total	4529	100

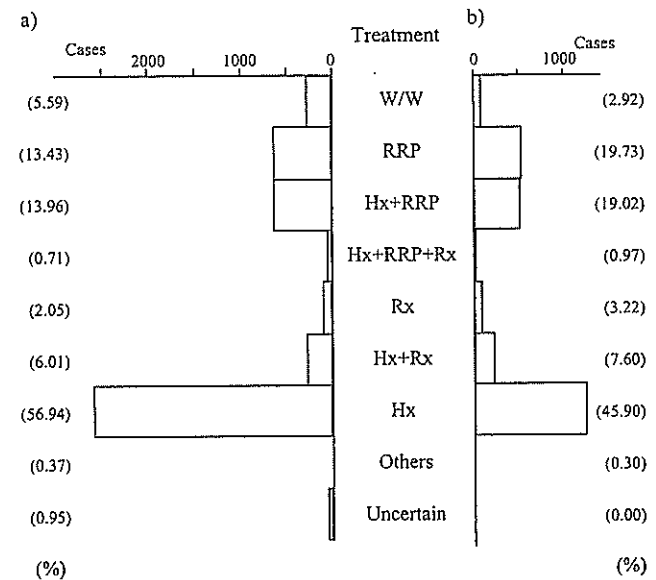


Fig. 5 Initial treatment plan for (a) all registered cases ($n = 4529$) and (b) cases of T1c to T3 without lymph node or distant metastases ($n = 2671$). Registered cases were divided into groups by initial treatment plan, which includes each treatment modality and appropriate combinations, as indicated. W/W, watchful waiting; RRP, retro-pubic radical prostatectomy; Hx, hormonal therapy; Rx, irradiation. Bars indicate the number of cases in each group. The numbers below each bar indicate the percentage of cases in each group among all registered cases or the subgroup of patient at stage T1c to T3 without lymph node or distant metastases.

45.90% (1226 cases) were scheduled to undergo hormonal therapy without any other additional treatment. Irradiation was not commonly advised for this group (10.8%).

Additional data are summarized in Appendix I-IV.

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Appendix I. General statistics

I.1. Background

I.1.1. Races

Race	Number	%
Japanese	4509	99.56
Mongolian (without Japanese)	13	0.29
White	6	0.13
Uncertain	1	0.02
Total	4529	100

I.1.3. Occupation

Occupation	Number	%
Service workers	101	2.23
Transportal and postal related workers	63	1.39
Corporate and general managers	314	6.93
Craft, related trades workers and elementary occupations	165	3.64
Mining labourers	8	0.18
Office clerks and associate professionals	365	8.06
Professionals and technicians	300	6.62
Agricultural, forestry and fishery workers	304	6.71
Sales workers	119	2.63
Protective services workers	14	0.31
Others or retired	1002	22.12
Uncertain	1774	39.17
Total	4529	100

I.2. Symptoms

I.2.1. Urological

Symptoms	Number	%
+	3219	71.08
-	1273	28.11
Uncertain	37	0.82
Total	4529	100

I.3. Staging method and findings

I.3.1. Modality of image study

Number	% in all cases	
CT	3023	66.75
MRI	1937	42.77
Bone scan	3330	73.53
TRUS	3547	78.32

I.1.2. Family history of prostate cancer

	Number	%
Father	30	0.66
Brother	37	0.82
Uncle	4	0.09
Total	71	1.57

I.1.4. Distribution of organs of concomitant malignancy

Organ	Number
Kidney	33
Urothelial	111
Testis	2
Penile	3
Head and neck	34
Lung	50
Esophageal	10
Stomach	107
Colon	120
Liver and gall bladder	19
Pancreas	9
Skin	6
Hematological	8
Sarcoma	3
Breast	1
Uncertain and other	4
Total	520
Negative	4022

I.2.2. Metastatic

Symptoms	Number	%
+	339	7.49
-	4137	91.34
Uncertain	53	1.17
Total	4529	100

I.4. Pathological findings in biopsy specimens**I.4.1. Histological type**

Pathological type	Number	%
Adenocarcinoma	4519	99.80
Neuroendocrine tumor	3	0.10
Transitional cell carcinoma	2	0.00
Other	2	0.00
Uncertain	3	0.10
Total	4529	100

I.4.3. Poorer differentiation

Number	%	
W/D	959	21.20
M/D	1664	36.70
P/D	1337	29.50
Uncertain	569	12.60
Total	4529	100

I.5. TNM classification**I.5.1. T stage distribution**

T stage	Number	%
T0	12	0.27
T1a	166	3.73
T1b	78	1.75
T1c	901	20.26
T2a	968	21.77
T2b	771	17.34
T3a	702	15.79
T3b	491	11.04
T4	358	8.05
Total	4447	100

TNM stage followed by 1997 UICC's TNM classification.

I.5.2. N stage distribution

N stage	Number	%
N0	3569	78.80
N1	501	11.06
NX	459	10.13
Total	4529	100

I.4.2. Predominant differentiation

Pathological pattern	Number	%
W/D	1416	31.30
M/D	1972	43.50
P/D	931	20.60
Uncertain	210	4.60
Total	4529	100

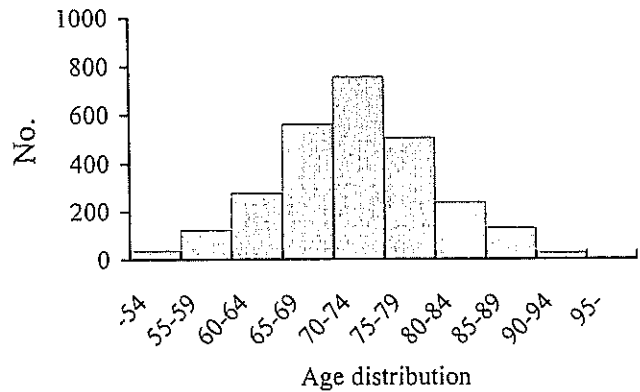
I.5.3. M stage distribution

M stage	Number	%
M0	3243	71.61
M1a	40	0.88
M1b	861	19.01
M1c	63	1.39
MX	322	7.11
Total	4529	100

II. General statistics in clinical T1c-T3N0M0 prostate cancer

II.1. Age distribution

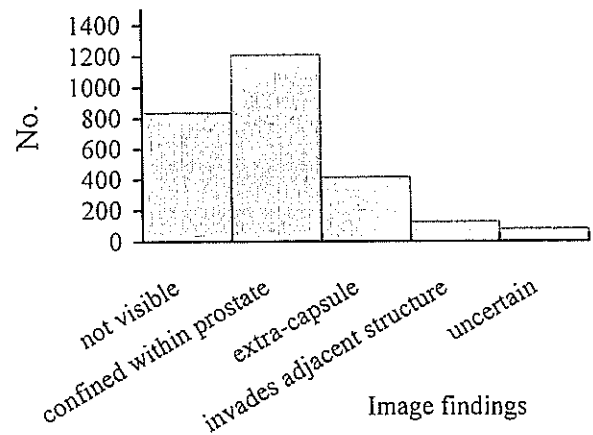
Age range	Number	%
<54	38	1.42
55-59	123	4.61
60-64	279	10.45
65-69	562	21.04
70-74	756	28.30
75-79	506	18.94
80-84	240	8.99
85-89	132	4.94
90-94	27	1.01
95>	8	0.15
Total	2671	100



Cases were divided into age groups by 5 years as indicated. Bars demonstrate the number of cases between equal and over the age on the left lower corner and below the age on the right lower corner of each bar. The numbers below each bar indicates the percentage of cases in each age group among all registered cases.

II.2. Findings of image study

Number	%	
Not visible	836	31.87
Confined within prostate	1209	46.09
Extra-capsule	416	15.86
Invades adjacent structure	128	4.88
Uncertain	82	1.30
Total	2671	100



II.3. DRE findings

Number	%	
Not palpable	1174	43.95
One lobe	786	29.43
Both lobe	362	13.55
Extra-capsule	215	8.05
SV invasion	40	1.50
Extra-prostate	5	0.19
Uncertain	89	3.33
Total	2671	100

