$P = 0{\cdot}007$]. TRM was analysed in the subset of patients with standard-risk malignancy in relation to UGT2B17 genotype in the donor by the Kaplan–Meier method (Fig 1A). TRM in the patients transplanted from an UGT2B17-deleted donor was markedly lower than in those transplanted from an UGT2B17-positive donor (23·7% vs. 52·9%; $P = 0{\cdot}001$).

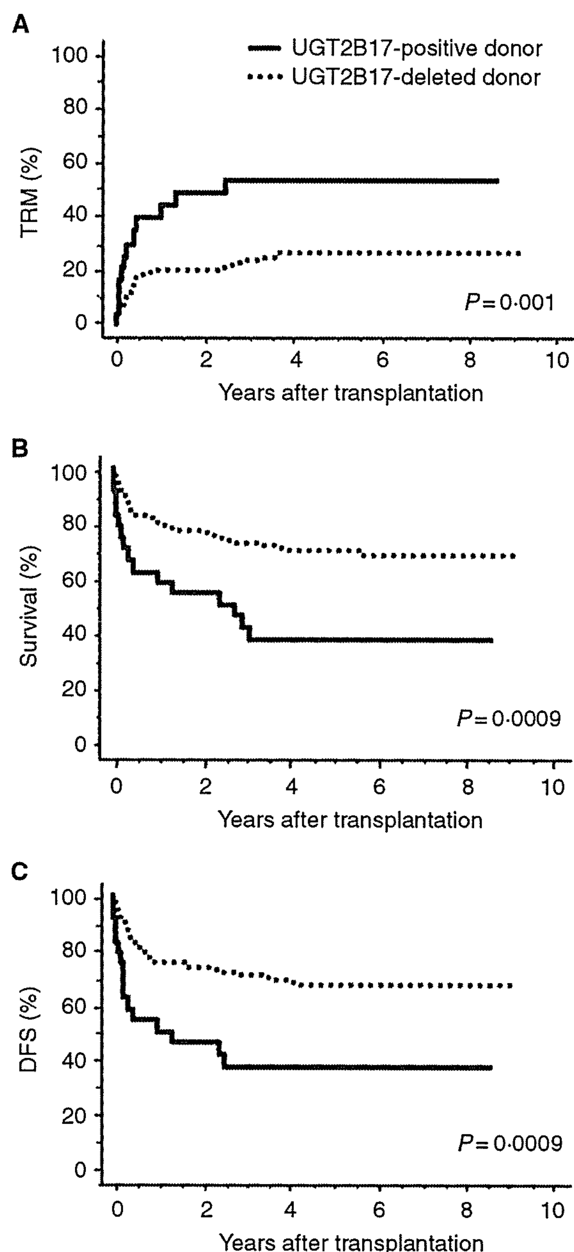

Fig 1. Impact of homozygous deletion of *UGT2B17* on TRM, survival and DFS in the subset of patients with standard-risk of malignancy. (A) TRM in the patients transplanted from UGT2B17-positive donor was 52·9% ($n = 24$) and that from UGT2B17-deleted donor was 23·7% ($n = 120$). (B) Survival in those two groups was 37·5% ($n = 24$) and 68·3% ($n = 119$), respectively, and (C) DFS was 37·5% ($n = 24$) and 66·9% ($n = 120$), respectively.

Seventy-five patients with malignancy who were transplanted from an UGT2B17-deleted donor died while they were in remission. Causes of death in the patients were rejection/graft failure ($n = 4$; 5%), GVHD ($n = 12$; 16%), interstitial pneumonia ($n = 17$; 23%), sepsis ($n = 11$; 15%), bleeding ($n = 5$; 7%), liver failure ($n = 5$; 7%), renal failure ($n = 2$; 3%), thrombotic microangiopathy ($n = 2$; 3%), veno-occlusive disease ($n = 2$; 3%) and others ($n = 15$; 20%). Twenty-four patients with malignancy who were transplanted from an UGT2B17-positive donor died while they were in remission. Causes of death in the patients were rejection/graft failure ($n = 1$; 4%), GVHD ($n = 3$; 13%), interstitial pneumonia ($n = 9$; 37%), sepsis ($n = 5$; 21%), bleeding ($n = 2$; 8%) and others ($n = 4$; 17%). There was no significant difference in the frequencies of each cause of death between the patients transplanted from UGT2B17-deleted donor and those from UGT2B17-positive donor.

Survival and DFS

Of the 364 evaluable patients with malignant disease, 190 (52%) were alive at the time of survey and 180 (49%) were alive in remission. In a univariate analysis, higher patient age, advanced disease, grades II–IV acute GVHD and grades III–IV acute GVHD were significantly associated with lower survival and DFS (Table II). In a multivariate analysis, the association of higher patient age, advanced disease and grades III–IV acute GVHD with lower survival and DFS remained significant.

Similar to the results with TRM, UGT2B17 deletion in the donor was a favourable factor for survival (relative risk, 0·57; 95% CI, 0·39–0·83; $P = 0{\cdot}004$) and DFS (0·65; 0·44–0·95; $P = 0{\cdot}024$). In the subset of patients with standard-risk malignancy analysed by the Kaplan–Meier method, both survival and DFS were significantly higher in patients transplanted from a UGT2B17-deleted donor than those transplanted from a UGT2B17-positive donor (survival, 68·3% vs. 37·5%, $P = 0{\cdot}0009$; DFS, 66·9% vs. 37·5%, $P = 0{\cdot}0009$) (Fig 1B and C).

Discussion

T-cell responses to minor H antigens that are broadly expressed have been implicated in GVHD after HLA-identical HSCT (Warren *et al*, 1998). The identification of minor H antigens that are associated with GVHD could potentially improve donor selection and identify recipients who are at high risk for GVHD. In this study, we focused attention on the *UGT2B17* gene that maps to chromosome 4q13 and has been identified to encode a minor H antigen presented by HLA-A29. A T-cell response to UGT2B17 was associated with GVHD after transplantation of an UGT2B17-positive recipient with stem cells from a donor with a homozygous deletion of the *UGT2B17* gene (Murata *et al*, 2003). Although homologous to other UGT2B family members, the UGT2B17 protein contains polymorphic sequences in addition to the region containing

the HLA-A29 epitope, including sequences that are predicted to bind to other class I HLA alleles and could provide additional minor H antigens. Therefore, we examined in a population study whether the transplant of an UGT2B17-positive recipient from an UGT2B17-deleted donor was associated with a higher incidence of GVHD.

Genotyping of unrelated individuals in Japan who served as transplant donors revealed a frequency of homozygous deletion of *UGT2B17* of 85%, which was substantially higher than the frequency of 11% observed in White people (Murata *et al*, 2003). A study of another UGT, UGT1A1 showed that the frequency of UGT1A1*28 allele was 29% in White people but only 9% in Japanese (Hall *et al*, 1999; Kohle *et al*, 2003). These striking differences in genotypes in different ethnic groups suggest that the components of the UGT enzyme family are quite distinct in different races.

The present study revealed no statistically significant association between UGT2B17 mismatch (UGT2B17-deleted donor and UGT2B17-positive recipient) in the GVHD direction and a higher incidence of GVHD. Several reasons may explain the lack of an association with GVHD. First, it is unknown how many of the polymorphic UGT2B17 peptides that were predicted by computer algorithm are actually processed and presented on the cell surface for potential recognition as a minor H antigen. Secondly, there may be an unidentified UGT2B family member whose amino acid sequence is homologous to UGT2B17. The UGT2B family presently consists of seven members, 2B4, 2B7, 2B10, 2B11, 2B15, 2B17 and 2B28 (Levesque *et al*, 2001; Belanger *et al*, 2003). If an unidentified UGT2B member encodes peptides with the same amino acid sequence as the corresponding UGT2B17 peptides, the UGT2B17 determinant could not serve as a minor H antigen. Thirdly, there is likely to be a large number of unknown minor H antigens encoded by other genes that are mismatched between each unrelated donor and patient pair and the effect of a UGT2B17 might be obscured in this setting. Fourthly, it is possible that the HLA-DPB1 allele was disparate between some donor and patient pairs, and this might obscure the effect of UGT2B17 mismatch (Petersdorf *et al*, 2001). Thus, the absence of a significant association between a UGT2B17 mismatch and GVHD in a population study does not exclude the possibility that UGT2B17 is a target of GVHD in selected patients.

Only a few minor H antigens have been studied for their contributions to the graft-*versus*-host reactions after allogeneic HSCT (Bleakley & Riddell, 2004). The autosomal minor H antigen HA-1 is the most extensively studied for its association with GVHD after HLA-identical stem cell transplantation and contradictory results have been reported (Goulmy *et al*, 1996; Tseng *et al*, 1999; Murata *et al*, 2000; Gallardo *et al*, 2001; Lin *et al*, 2001). For HA-8, which is ubiquitously expressed in tissues, a single study showed a significantly increased risk of grades II–IV acute GVHD in HA-8-positive recipients who received transplants from HA-8-negative donors, although the odds ratio of 1·8 (95% CI, 1·0–3·1) was small (Akatsuka *et al*, 2003). Discordance between donors and recipients at the ACC-1 minor H antigen was analysed for association with transplant outcome after HLA-identical unrelated bone marrow transplant. Although ACC-1 is restricted to haematopoietic cells, including leukaemia cells, there was no statistically significant association between ACC-1 mismatch in the graft-*versus*-leukaemia direction and lower rate of relapse after transplantation (Nishida *et al*, 2004). These results suggest that an analysis of discordance at a single minor H antigen locus in an outbred human population may have a limited ability to detect an association between a minor H antigen mismatch and GVHD or a graft-*versus*-leukaemia effect.

A surprising finding in our study was that transplantation from donors with a homozygous deficiency of *UGT2B17* was associated with a significantly lower risk of TRM and superior overall survival and DFS. UGT2B17 is an enzyme that is highly expressed not only in the liver and gastrointestinal tract, but also in subsets of haematopoietic cells. The enzyme is important in androgen metabolism and also serves to conjugate a variety of drugs, dietary components, and toxic exogenous compounds (Tukey & Strassburg, 2000). Some exogenous compounds were recently analysed as potential substrates for UGT2B17, however drugs used in allogeneic HSCT, such as chemotherapeutic agents, immunosuppressants, antibiotics, antiviral and antifungal drugs, have not been extensively analysed (Turgeon *et al*, 2003). Another member of the UGT2B family, UGT2B7, has been shown to glucuronidate CsA and tacrolimus (Strassburg *et al*, 2001). UGT2B17 is 88% identical to UGT2B7 at the amino acid level, and it is conceivable that UGT2B17 plays a role in the metabolism of these immunosuppressive drugs. We have previously shown that UGT2B17 RNA and protein are both expressed in haematopoietic cells including B cells and dendritic cells (Murata *et al*, 2003), and others have shown glucuronyltransferase activity in lymphocytes (Gessner *et al*, 1978; Li *et al*, 1981). Glucuronidation in lymphocytes may not make a major quantitative contribution to the overall metabolism of a compound in the body, but might increase the local deactivation of pharmacologically active compounds in the cell. The data in this study suggests the presence of UGT2B17 in donor-derived haematopoietic cells increases the susceptibility of the recipient to TRM. The increased mortality could not be attributed to a particular increase in GVHD, infections, other complications or relapse, and was observed in the subset of patients with standard risk malignancy. This observation suggests that additional study of the role of UGT2B17 in haematopoietic cells is warranted.

In summary, we studied the effects of homozygous deletion of *UGT2B17* gene with outcome after allogeneic unrelated donor HSCT. The use of an UGT2B17-positive donor was an independent risk factor for higher TRM and lower survival and DFS. Given that *UGT2B17* encodes a glucuronosyltransferase enzyme, it is possible the enzymatic function of this protein in haematopoietic cells impacts TRM and survival. Further analysis in a larger study population is warranted, and if our

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findings are confirmed, it may be important to consider UGT2B17 genotype in donor selection strategies.

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Identification of *CCND3* and *BYSL* as Candidate Targets for the 6p21 Amplification in Diffuse Large B-Cell Lymphoma

Yumiko Kasugai,[1] Hiroyuki Tagawa,[1] Yoshihiro Kameoka,[1] Yasuo Morishima,[2] Shigeo Nakamura,[3] and Masao Seto[1]

Abstract

Purpose: Increases in gene dosage through DNA amplification represents a common feature of many tumors and can result in the up-regulation of tumor-promoting genes. Our recent genome-wide, array-based comparative genomic hybridization analysis of 66 cases of diffuse large B-cell lymphoma found that genomic gain of 6p21 was observed in as many as 17 cases, including 14 cases with low-level copy number gain and three cases with high-level copy number gains (amplifications).

Experimental Design and Results: To identify the target gene(s) for 6p21 amplification, we constructed a detailed amplicon map at the region of genomic amplification with the aid of high-resolution contig array-based comparative genomic hybridization glass slides, consisting of contiguously ordered bacterial artificial chromosome/P1-derived artificial chromosome clones covering 3 Mb throughout the 6p21 amplification region. Alignment of the amplifications identified a minimally overlapping 800 kb segment containing 15 genes. Quantitative expression analysis of the genes from both patient samples and the SUDHL9 cell line revealed that *CCND3* and *BYSL* (1.9 kb telomeric to the *CCND3* gene locus) are the targets of 6p21 genomic gain/amplification.

Conclusions: Although it is known that t(6;14)(p21;q32) induces aberrant overexpression of *CCND3* in B-cell malignancies, we were able to show that *CCND3*, which encodes the cyclin D family member protein that controls the G_1-S phase of cell cycle regulation, can also be a target of genomic gain/amplification. Overexpression of *CCND3* through genomic amplification is likely to lead to aberrant cell cycle control, although the precise biological role of *BYSL* with respect to tumorigenesis remains to be determined.

Deregulation of oncogenes via genomic amplifications is a common occurrence in various tumors, including malignant lymphomas. Previous studies have reported several candidate genes of genomic amplification in malignant lymphomas, such as 2p15 amplification with *REL* overexpression (primary large B-cell lymphomas of the gastrointestinal tract, Hodgkin lymphoma; refs. 1-3), 9p24 amplification with *JAK2* and/or *PDL2* overexpression (primary mediastinal B-cell lymphoma, Hodgkin lymphoma; refs. 4-6), and 10p12 amplification with *BMI-1* overexpression (mantle cell lymphoma; ref. 7). Very recently, we showed that *C13orf25*, which includes a micro-RNA cluster, was overexpressed in association with the 13q31-q32 genomic amplification in various B-cell lymphomas (8). However, the majority of the genes responsible for genomic amplification remain to be identified.

Our recent array-based comparative genomic hybridization (array CGH) study of diffuse large B-cell lymphoma (DLBCL) has identified recurrent high-level genomic aberrations as 1q31-q32, 2p15, 6p21, 9p24, 11q22-q24, 13q31, and 18q21 (9). Of these genomic alterations, the various cytogenetic abnormalities of chromosome band 6p21 in mature B-cell malignancies include translocations and amplifications. t(6;14)(p21;q32) has been previously reported in a variety of B-cell malignancies, such as DLBCL and splenic marginal zone lymphomas, and it has been shown that deregulation of *CCND3* is a result of this translocation (10).

Although recurrent amplifications of 6p21 have been detected and described in B-cell lymphomas, such as follicular cell lymphoma, mantle cell lymphoma, and DLBCL (11-13), no detailed studies have been conducted of the gene(s) responsible for the amplification. To further identify these target gene(s) in DLBCL, we did the "contig" array CGH using

Authors' Affiliations: [1]Division of Molecular Medicine, Aichi Cancer Center Research Institute, Departments of [2]Hematology and Cell Therapy and [3]Pathology and Molecular Diagnostics, Aichi Cancer Center Hospital, Aichi, Japan

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Note: Y. Kasugai and H. Tagawa equally contributed to this work and share with the first authorship.

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Requests for reprints: Masao Seto, Division of Molecular Medicine, Aichi Cancer Center Research Institute, 1-1 Kanoko-den, Chikusa-ku, 464-8681 Nagoya, Japan. Phone: 81-52-762-6111, ext. 7080/7082; Fax: 81-52-764-2982; E-mail: mseto@aichi-cc.jp.

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glass slides on which contiguously ordered bacterial artificial chromosome/P1-derived artificial chromosome (BAC/PAC) clones were spotted throughout 3 Mb of the 6p21 genome.

Materials and Methods

Tumor samples and B-cell lymphoma cell lines. Data of genomic gains and losses region of 66 DLBCL cases have been reported previously (9). The cell lines used in the study presented here were SUDHL9 (Southwestern University: diffuse large B-cell lymphoma cell line), SP49 (mantle cell lymphoma cell line; ref. 14), and OCI-LY8 (immunoblastic B-cell lymphoma; ref. 15). These cell lines were maintained in RPMI 1640 supplemented with 10% FCS at 37°C.

DNA and RNA samples. DNA was extracted with a standard phenol-chloroform method from lymphoma specimens of tumors and of SUDHL9, SP49, and OCI-LY8. Normal DNA was prepared from peripheral blood lymphocytes of healthy male donors. Total RNA was extracted with the standardized guanidium isothiocyanate and cesium chloride method from human placenta and normal lung as well as from SUDHL9, SP49, and OCI-LY8.

Fluorescence in situ *hybridization and comparative genomic hybridization analyses.* Fluorescence *in situ* hybridization (FISH) and CGH were done as described elsewhere (8).

Genome-wide array-based comparative genomic hybridization. DNA preparation, labeling, array fabrication, and hybridization were done as described elsewhere (8, 9, 16). Briefly, the array consisted of 2,088 BAC and PAC clones, covering the human genome at a 1.5 Mb resolution, from library RP11 and RP13 for BAC clones and RP1, RP3, RP4, and RP5 for PAC clones. Of the 2,088 clones spotted on the glass slides, 121 were of chromosome 6; of the 121 BAC/PAC clones, 50 were of the short arm of chromosome 6 (6p). These clones were obtained from the BAC/PAC Resource Center at the Children's Hospital Oakland Research Institute in Oakland, CA (http://bacpac.chori.org/). The thresholds for the $\log_2$ ratio of gains and losses were set at $\log_2$ ratios of +0.2 and −0.2, respectively. High-level gain (amplification) was defined as $\log_2$ ratio $\geq +1$ and low-level copy number gain was defined as $+0.2 \leq \log_2$ ratio $< +1.0$ (8).

Contig array-based comparative genomic hybridization. Twenty-five BAC/PACs of 6p21 were isolated from their bacterial cultures with the relevant antibiotics and extracted with a plasmid mini kit (Qiagen, Valencia, CA). The exact location of each clone was determined by standard FISH analysis. Degenerate oligonucleotide primed PCR (17) was done on the DNA of BAC/PAC clones as described before (8). Degenerate oligonucleotide primed PCR products were dissolved in 30 μL of TE buffer [100 mmol/L Tris-HCl and 1 mmol/L EDTA (pH 7.5)], and 10 μL of Solution I (Takara Bio, Inc., Tokyo, Japan) was added to each of the products, which were then spotted in triplicate onto the Hubble-activated slides (Takara Bio) using the Stampman Arrayer (Nippon Laser and Electronics Lab, Nagoya, Japan) with a split pin. Slides were fixed in 0.2% SDS for 2 minutes and in 0.3 N NaOH for 5 minutes, then dehydrated with 100% cold ethanol for 3 minutes, and finally air dried. DNA preparation, labeling, array fabrication, and hybridization were done according to the method described previously (8, 9, 18, 19).

Image scanning. The Agilent Micro Array Scanner (Agilent Technologies, Palo Alto, CA) was used for scanning analysis. The array images thus acquired were analyzed with the Genepix Pro 4.1 (Axon Instruments, Inc., Foster City, CA).

Reverse transcription-PCR analysis for screening of candidate genes. Human placenta, normal lung, SUDHL9, SP49, and OCI-LY8 were subjected to reverse transcription-PCR (RT-PCR) analysis, whereas SuperScriptII (Life Technologies, Division of Life Technologies, Inc., Gaithersburg, MD) was used for cDNA derived from human placenta and normal lung. Each 5 μg of total RNA was reverse-transcribed into cDNA dissolved in 40 μL of distilled water. RT-PCR was done for 25 genes using the specific corresponding primers. Gene names and accession numbers were as follows: *FOXP4* (NM_138457), *MDF1* (NM_005586), *TFEB* (NM_007162), *PGC* (NM_002630), *FRS3* (NM_006653), *C6orf49* (NM_013397), *USP49* (NM_004275), *BYSL* (NM_004053), *CCND3* (NM_001760), *TBN* (NM_138572), *LOC389389* (XM_371820 XP_371820), *GUCA1A* (NM_000409), *GUCA1B* (NM_002098), *MRPS10* (NM_018141), *TRERF1* (AF297872, AL096814), *C6orf133* (NM_015255), *RDS* (NM_000322), *TBCC* (NM_003192), *KIAA0240* (XM_166479 XP_166479), *RPL7L1* (NM_198486), *PTCRA* (NM_138296), *TNRC5* (NM_006586), *LOC389390* (XM_374167 XP_374167), *GNMT* (AF101477), and *PEX6* (NM_000287). Each primer was designed so that the T_m value would be between 55°C and 60°C. Amplifications were done on a Thermal Cycler (Perkin-Elmer Corporation, Norwalk, CT), and RT-PCR was done with the touchdown PCR method. The reactions comprised 10 cycles of denaturation (94°C, 0.5 minutes), annealing (63°C, 0.5 minutes, 1°C decrease per two cycles), and extension (72°C, 2.5 minutes), followed by 35 cycles of denaturation (94°C, 0.5 minute), annealing (58°C, 0.5 minute), and extension (72°C, 2.5 minutes), and a final extension of 5 minutes at 72°C. Basically, the annealing temperature of the reaction ranged from 63°C to 58°C. RT-PCR was also done under different conditions by changing the annealing temperature from 65°C to 60°C or from 60°C to 55°C. If no PCR products were obtained, we designed new primer sets to confirm the negativity of genes. All PCR products were separated by electrophoresis and purified using the QIA quick Gel Extraction kit (Qiagen). Direct sequence determination with the same primers used for nested-PCR was done with an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, VA). DNA sequences were compared with those in the Genbank databases with the aid of the BLAST program available at web site, http://www.ncbi.nlm.nih.gov.

Northern blot analysis. Several cell lines and normal tissues were subjected to Northern blot analysis. Probes were radiolabeled with a random primer DNA labeling kit (Nippon Gene, Tokyo, Japan) with [α-^{32}P]dCTP. Total cellular RNA (5 μg) was size-fractioned on 1% agarose/0.66 mol/L formaldehyde gel and transferred onto a Hybond-N$^+$ nylon membrane (Amersham Pharmacia Biotech, Tokyo, Japan). The membranes were then hybridized overnight at 42°C with [α-^{32}P]dCTP-labeled probes, washed, and exposed to BIOMAX MS films (EKC, Rochester, NY). All the genes were equally exposed for 24 hours on BIOMAX films at −20°C after hybridization. Densitometric scanning for radiographical signals was done by an ImageMaster VDS-CL (Amersham Pharmacia Biotech).

Quantitative real-time reverse transcription-PCR. Expression levels of *CCND3* and *BYSL* mRNA were measured by means of real-time fluorescence detection using a previously described method (20). Briefly, the primers of *CCDN3* were sense: 5′-GACCGACAGGCCTTGGTCAA-3′ and antisense: 5′-AGTGCCAGTGATCCCTGCCA-3′, and those of *BYSL* were sense: 5′-AGAAGGCTGCCACAATGACA-3′ and antisense: 5′-GACATGACTGTCTCAACCTC-3′. The real-time PCR using CYBR Green and the primers was done with a Smart Cycler System (Takara Bio) according to the protocol of the manufacturer. *G6PDH* served as an endogenous control, whereas the expression levels of *CCDN3* and *BYSL* mRNA in each sample were normalized on the basis of the corresponding *G6PDH* content and recorded as relative expression levels.

Statistical analysis. The Mann-Whitney *U* test was done for detecting significance in expression levels of *CCND3* and *BYSL* between groups with and without 6p21 genomic amplifications. All the statistical analyses were conducted with the STATA version 8 statistical package (StataCorp, College Station, TX).

Results

Recurrent high-level amplification at 6p21 in diffuse large B-cell lymphoma. The array CGH analysis at a resolution of 1.5 Mb throughout the whole genome showed that 26 of 66 DLBCL cases had copy number gains on chromosome 6p (Fig. 1). Seventeen of the 26 cases included 6p21 gain, with 3 of

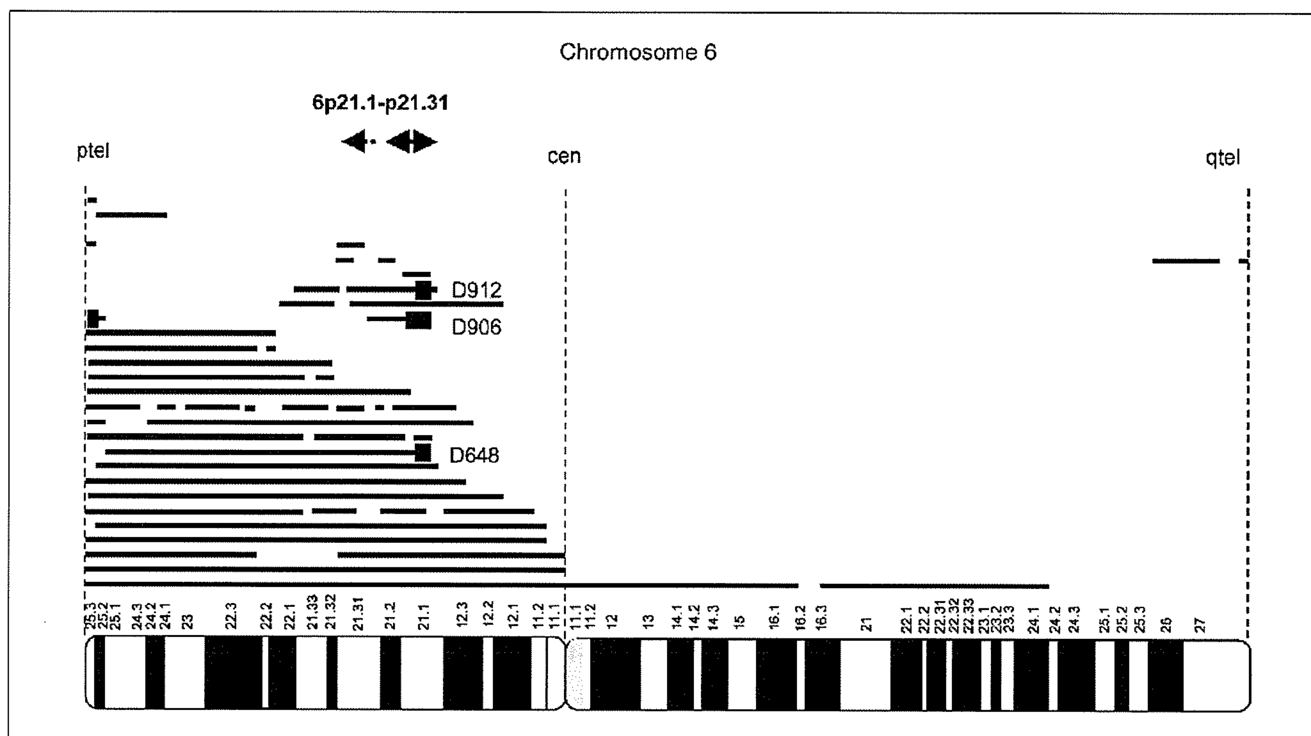

Fig. 1. Schematic illustration of genomic amplification at chromosome 6. These data were obtained by genome-wide array CGH analysis of 66 DLBCL cases. Thin lines, low copy number gain ($+0.2 \le \log_2$ ratio $< +1.0$); thick lines, high copy number gain (amplifications; $\log_2$ ratio $\ge +1.0$). Genomic amplifications were observed at 6p21 in three cases and at 6p25 (*IRF4* locus) in one case.

the 17 cases showing genomic amplification ($\log_2$ ratio >1) at PAC, RP5-973N23. As shown in Fig. 2, partial genomic profiles of three individual tumors (D906, D648, and D912) and the SUDHL9 cell line showed that each of the highest peaks was detected at PAC, RP5-973N23, indicating that the peaks may be biologically significant. FISH analysis using PAC, RP5-973N23 as the probe confirmed strong genomic amplification at 6p21 in SUDHL9 (>15 copies; Fig. 3). The amplification-overlapping region of the three tumors (D906, D648, and D912) and SUDHL9 could be clearly defined to the restricted region between BAC, RP11-552E20 (40.3 Mb) and PAC, RP5-895C5 (43.5 Mb) at 6p21.

Determination of amplicon core by contig array comparative genomic hybridization. We speculated the target genes of the high copy number gains of 6p21 were within the 3 Mb region between 40.3 Mb (BAC, RP11-552E20) and 43.5 Mb (PAC, RP5-895C5). To specify the alterations of 6p21 in greater detail, we constructed high-resolution contig array glass slides containing 25 BAC/PAC clones, which were contiguously placed throughout the 3 Mb region at the 6p21 genome. Contig array CGH was conducted for the SUDHL9 cell line and three tumors (D906, D648, and D912), for which the genome-wide array CGH showed high copy number gains at 6p21. The analysis was also conducted for OCI-LY8 and SP49 cell lines that did not show genomic amplification at chromosome 6. Mixed partial individual genomic profiles of 6p21 for D906, D648, D912, SUDHL9, and OCI-LY8 are shown in Fig. 4. It was found that the "amplicon core" region of 6p21 was narrower than could be expected from data obtained from the genome-wide array CGH. Contig array CGH showed that the minimal common region (amplicon core region) of the three tumors and SUDHL9 was 800 kb in length and ranged from BAC, RP11-328M4 (41.6 Mb) to PAC, RP1-139D8 (42.3 Mb). The amplicon core contained 15 genes and it was speculated that candidate genes of 6p21 amplification were located within this region.

Reverse transcription-PCR analysis of the genes within the 6p21 amplification region of SUDHL9. The contig array CGH for SUDHL9 showed that 25 known genes reside within the 6p21 amplification region (2.5 Mb in length), which includes the "amplicon core." Fifteen of these genes lie within the amplicon core, whereas the other 10 are localized centromerically to it (Fig. 4). RT-PCR using human placenta, normal lung, SUDHL9, SP49, and OCI-LY8 cell lines was used to screen for expressions of these 25 genes. The genes and primers used for RT-PCR and the results are shown in Supplemental Table S1. The expected sizes of all products obtained by RT-PCR were confirmed by electrophoresis. Expression of 13 genes (*FOXP4, TFEB, FRS3, USP49, BYSL, CCND3, TBN, MRPS10, TRERF1, TBCC, KIAA0240, PTCRA*, and *TNRC5*) could be detected in SUDHL9 but not of the other 12 genes. *MDF1* and *GUGA1A* did not result in any PCR products in the five RNA samples studied, and although the RT-PCR of *MDF1* and *GUGA1A* was done under different conditions by changing the annealing temperature from 65°C to 60°C or from 60°C to 55°C, and with different primer pairs to detect these genes, no bands were detected. We, therefore, concluded that the expression levels of these two genes were undetectable and excluded them for further expression analysis.

Northern blot analysis for screening gene expressions. Because expressions of the 13 genes were confirmed in SUDHL9 by RT-PCR analysis, we next did Northern blotting for quantitative

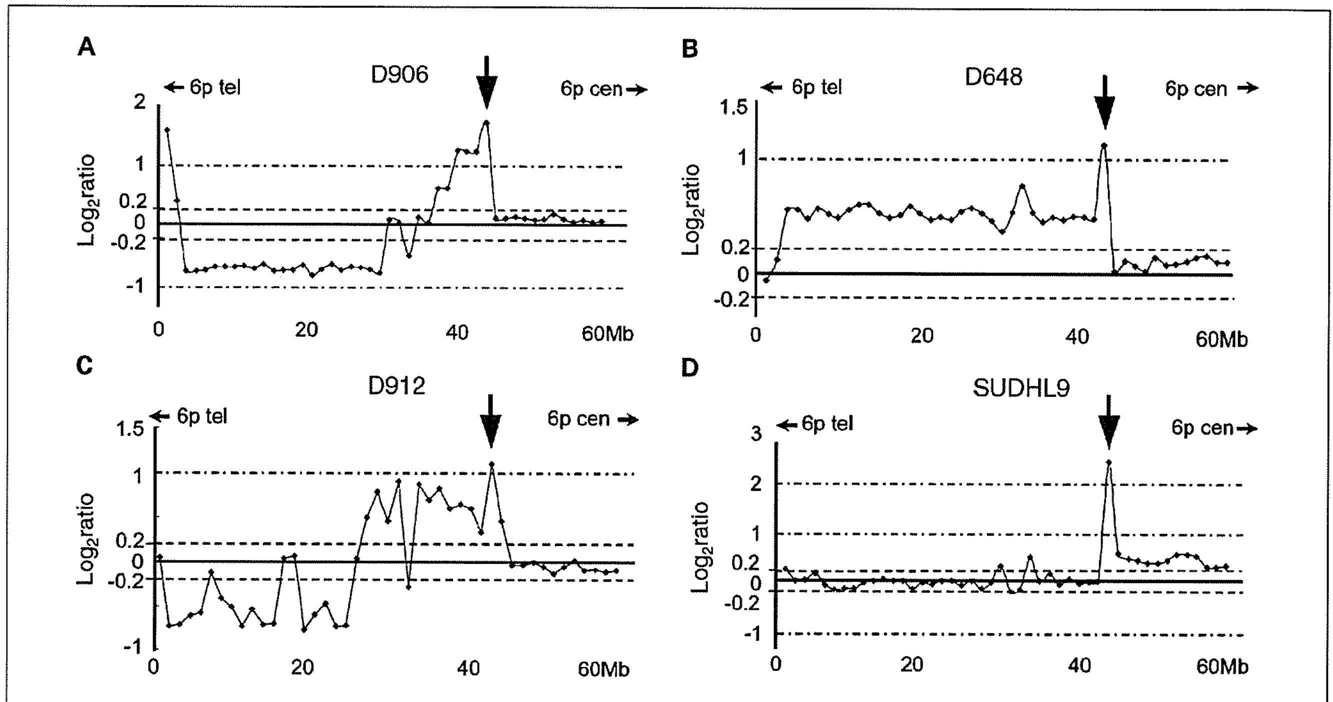

Fig. 2. Individual partial genomic profiles of chromosome 6p for three patient samples (D906, D648, and D912) and the SUDHL9 cell line. Horizontal lines, megabase from 6p telomere to centromere; vertical lines, $\log_2$ ratio. Each spot is contiguously ordered from p telomere to centromere with, on average, 1.5 Mb resolution. The threshold for gain and loss was defined as the $\log_2$ ratio of +0.2 and −0.2, respectively. *A*, D906; *B*, D648; *C*, D912; *D*, SUDHL9. Vertical thick arrow, highest peak at 6p21. Log_2 ratios: D906, 1.7; D648, 1.1; D912, 1.1; SUDHL9, 2.6.

analyses of gene expression in five samples (human placenta, normal lung, SUDHL9, SP49, and OCI-LY8). Expression levels of *BYSL, CCND3, TBN, TBCC,* and *KIAA0240* in SUDHL9 were on average 1.5 to 4 times higher than in human placenta, normal lung, SP49, and OCI-LY8 (Table 1). However, expressions of the other eight genes did not show good correlation with the level of genomic amplification. The possible candidate genes for 6p21 amplification were thus *BYSL, CCND3, TBN, TBCC,* and *KIAA0240*. To examine gene expressions of other hematologic malignancies, we conducted Northern blot analysis of these five genes for a variety of hematologic malignant cell lines that did not feature 6p21 amplification. These cell lines comprised five B-cell lymphomas, three T-cell lymphomas, one multiple myeloma, and two acute myeloid leukemias. Expression levels

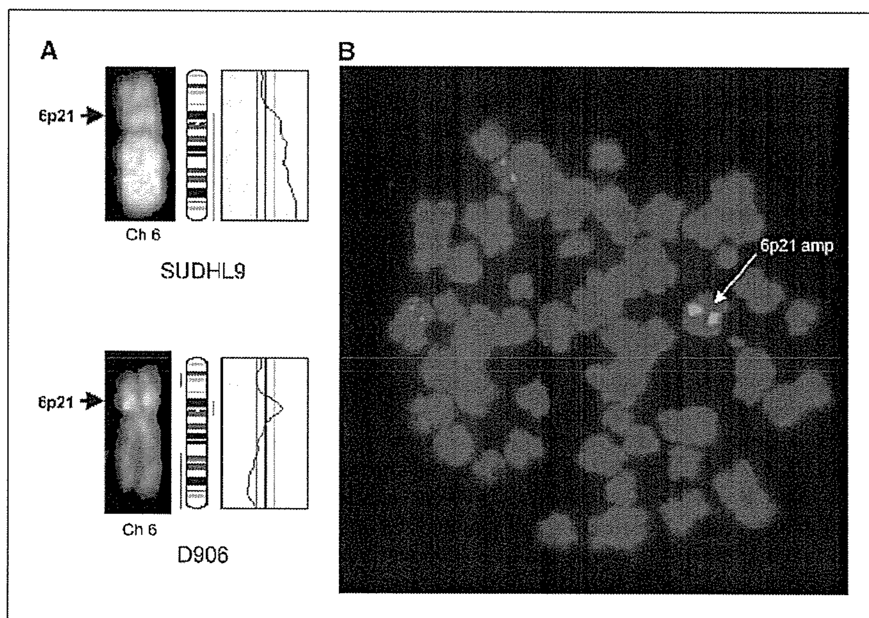

Fig. 3. CGH (*A*) and FISH (*B*) data of a patient sample (D906) and SUDHL9. *A*, conventional CGH. Conventional CGH accurately shows amplification at 6p21 in D906. Conventional CGH of SUDHL9 also shows genomic amplification at 6p21-qtel but fails to detect the amplicon at 6p21. *B*, FISH. RP5-973N23 (*red signal*) was the probe used for FISH analysis of the SUDHL9 cell line. Arrows, *6p21* locus.

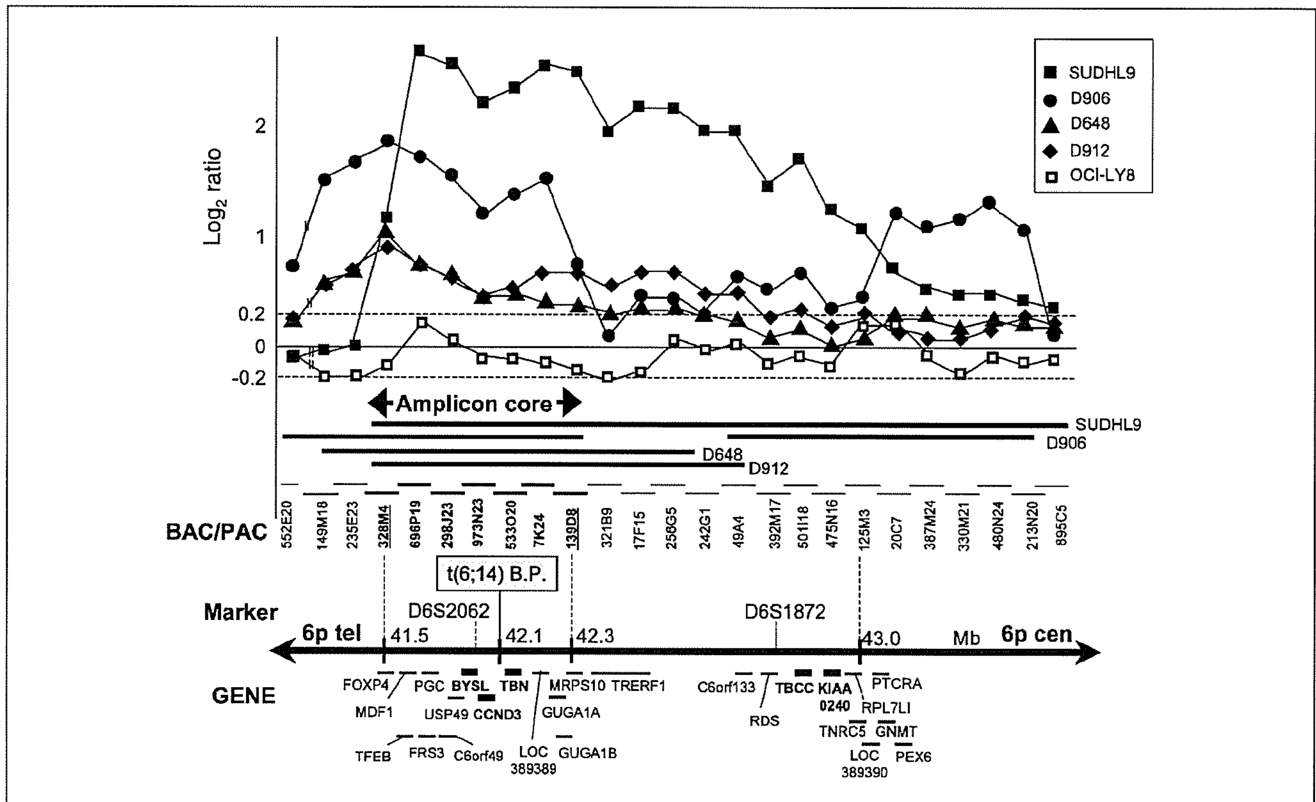

Fig. 4. 6p21 genomic profiles obtained with contig array CGH of three DLBCL patients (D906, D648, and D912) and two cell lines (OCI-LY8 and SUDHL9). Vertical line, $\log_2$ ratio. The threshold for gain and loss was defined as the $\log_2$ ratio of +0.2 and −0.2, respectively. Each 25th spot was contiguously placed from telomere to centromere according to its National Center for Biotechnology Information mapping position. tel, telomere; cen, centromere. BAC/PAC, sequence-tagged site markers, and gene symbols are given below the genome profile. The amplicon core is 800 kb long from BAC, RP11-328M4 (41.6 Mb) to PAC, RP1-139D8 (42.3 Mb).

of *BYSL*, *CCND3*, *TBN*, *TBCC*, and *KIAA0240* in the SUDHL9 cell line were again higher than in other cell lines (Fig. 5A).

Candidate gene identification by Northern blot analysis. Five patient samples, three of which possessed 6p21 amplifications, were subjected to Northern blot analysis. The expression level of *BYSL* in two patients (D906 and D912) with 6p21 amplifications was, on average, 1.7 times higher than that in patients without the amplification (Fig. 5B). Similarly, the

Table 1. Northern blot analysis of 6p21 candidate genes

Gene	Gene size (kb)	Probe size (bp)	Human placenta	Lung	SUDHL9	OCI-LY8	SP49
FOXP4	4.2	190	−	−	+	+	+
TFEB	2.5	190	++	+	+	+	++
FRS3	2.2	410	−	−	−	ND	−
USP49	2.4	440	++	++	++	ND	++
BYSL	1.3	300	+	+	+++	−	++
CCND3	2.0	2,000	−	+	+++	+/−	+
TBN	1.8	450	−	+/−	+	−	+
MRPS10	2.1	190	++	+	+	+	++
TRERF1	4	320	−	−	−	ND	−
TBCC	1.6	200	−	−	+	−	+/−
KIAA0240	7.1	210	−	−	+	−	+/−
PTCRA	1.1	140	−	−	−	ND	−
TNRC5	1.7	630	−	−	++	++	++

NOTE: The strength of the signal within each blot is represented from strongest (+++) to undetected (−). The mRNA expression level for each sample was normalized on the basis of the corresponding β-actin expression. Gene expression was normalized relative to β-actin (gene expression/β-actin): −, gene/β-actin < 0.25; −/+, 0.25 < gene/β-actin < 0.75; +, 0.75 < gene/β-actin < 1.25; ++, 1.25 < gene/β-actin < 1.75; +++, gene/β-actin > 1.75.
Abbreviation: ND, not done.

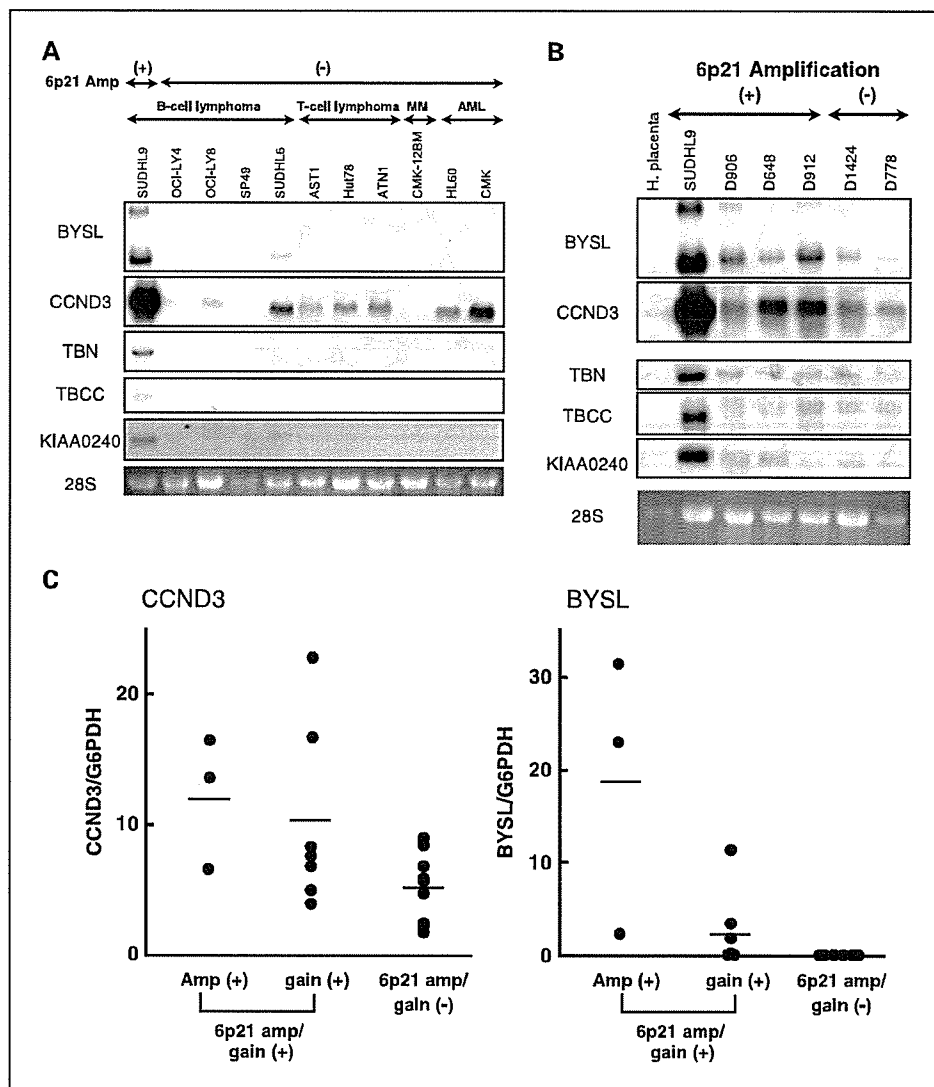

Fig. 5. Northern blot analysis of candidate genes. *A*, expression of five candidate genes of 6p21 amplification in various hematologic malignant cell lines. MM, multiple myeloma; AML, acute myeloid leukemia. Lane 1, SUDHL9; lane 2, OCI-LY4; lane 3, OCI-LY8; lane 4, SP49; lane 5, SUDHL6; lane 6, AST-1; lane 7, Hut78; lane 8, ATN1; lane 9, KMS-12BM; lane 10, HL60; lane 11, CMK. All samples were exposed under equal conditions for 24 hours on BIOMAX films at −20°C following hybridization. *B*, Northern blot analysis of candidate genes in five samples, three of which possessed 6p21 amplification. The expression level of *BYSL* and *CCND3* in patients with 6p21 amplification was on average 1.7 and 2.5 times higher than that in patients without 6p21 amplification, respectively. The expression of *TBN*, *TBCC*, and *KIAA0240* did not differ. *C*, quantitative real-time reverse transcription-PCR to detect the expression level of *CCND3* and *BYSL* in DLBCL patients with or without 6p21 gain/amplification. Twenty cases were divided into three groups with amplification (*6p21 amp +*), low copy number gains (*6p21 gain +*), and no copy number changes (*6p21 gain −*). Horizontal bars for each group, mean. Significantly higher expressions of *CCND3* ($P = 0.0343$) and *BYSL* ($P = 0.0082$) were observed in samples with (10 cases) rather than without (10 cases) 6p21 gain/amplification.

CCND3 expression level in two patients (D648 and D912) with 6p21 amplifications was on average 2.5 times higher than that in patients without the amplification. On the other hand, the expression level of *TBN*, *TBCC*, and *KIAA0240* did not differ between patients with or without 6p21 amplification. This suggested that the most likely candidate genes for 6p21 amplification are *BYSL* and *CCND3*. CCND3 and BYSL mRNA expression is higher in the SUDHL9 cell line than in patient cases. This might account for the higher $\log_2$ ratio of BAC RP5-973N23 in SUDHL9 ($\log_2$ ratio = 2.6), whereas the $\log_2$ ratio of patient cases was lower than the cell line ($1 < \log_2$ ratio < 2).

Quantitative real-time reverse transcription-PCR for CCND3 and BYSL. Quantitative real-time reverse transcription-PCR analysis of *CCND3* and *BYSL* was then done on 20 patient samples. As shown in Fig. 5C, the 20 DLBCL cases were divided into three groups with amplification (3 cases), low copy number gains (7 cases), and no copy number changes (10 cases). Samples derived from D648 and D912 with 6p21 amplification showed overexpression of *CCND3*. These two cases also showed overexpression of *BYSL*. The expression level of both *CCND3* and *BYSL* in cases that had shown low or high copy number gains (10 cases) was significantly higher than in cases without 6p21 gain (10 cases; *CCND3*, $P = 0.0343$; *BYSL*, $P = 0.0082$). These results lead us to conclude that the target genes of gain/amplification at 6p21 are *BYSL* and *CCND3*.

Discussion

Genomic amplification has been observed in a variety of tumors and represents one aberrant molecular pathway by which gene expression is constitutively enhanced beyond the level of physiologically normal variation. It can be expected that the "driver" genes are located at the narrow region with the highest level of copy number changes as shown in our study. Our strategy for the identification of target genes was to use analyses combining genome-wide (1.5 Mb resolutions throughout the genome) and contig array CGH (3 Mb in length at 6p21). Chromosome 6p may harbor several candidate oncogenes responsible for chromosome 6p gain/amplification, such as *IRF4* for 6p25 amplification and *E2F3*, *DEK*, and *RBKIN/KIF13A* that are associated with 6p22.3 gain. We were able to investigate candidate targets using three tumors and one cell

line that showed genomic amplification at the 6p21 region. We constructed a detailed 3 Mb physical map of the 6p21 amplicon, which included the 800 kb amplicon core. The structure of the 6p21 amplicon could be mapped in detail, and the number of copies throughout the amplified region was accurately estimated. The approach used by us and described here proved useful in characterizing amplified genomic regions of a wide variety of tumors, not only DLBCL. This strategy was also used in a previous study of ours in which we detected the aberrant expression of *FHIT* that had originated from a 3p14 small deletion in DLBCL (18).

Quantitative expression analyses showed that *BYSL* (21) and *CCND3* (22) are target genes of the amplicon core at 6p21, whereas *CCND3* is the translocation target of t(6;14)(p21.1;q32.3) in B-cell lymphoma (10). Moreover, *BYSL* and *CCND3* are located near each other. *CCND3* is centromeric to *BYSL*. Finally, the amplicon core includes the breakpoint of 6p21 translocation, and both *CCND3* and *BYSL* are generally telomeric to this breakpoint, indicating that both genes could be the targets for 6p21 chromosome translocation.

Although it has been widely speculated that the *CCND3* is the target gene for 6p21 genomic gain/amplification, no detailed investigations have been reported. The findings in the present report may, therefore, constitute the first evidence that *CCND3* is in fact the target for genomic gain/amplification in malignant lymphomas. Because CCND3 is the cyclin D family member protein that controls the G_1-S phase of cell cycle regulation, overexpression of *CCND3* through genomic amplification is likely to lead to aberrant cell cycle control and may contribute to tumorigenesis (23). Although *BYSL* is known as a bystin-like gene that mediates cell adhesion between trophoblasts and endometrial epitheral cells through its interaction with trophinin, tastin, and cytokeratine (21), the link between *BYSL* and tumorigenesis remains to be determined. It is likely that overexpression of *BYSL* results from its close proximity to *CCND3*. Interestingly, a similar co-overexpression pattern of two closely located genes has been reported in oral cancer cell lines by Huang et al. (24). They showed that the *TAOS1* gene, which is located ~12 kb distal to the *CCND1* gene, is co-overexpressed with *CCND1* with 11q13 amplification. Similarly, coexpression of *EMS1* with *CCND1* with 11q13 genomic amplification has been detected in several solid tumors (25-29). Although *EMS1* is known as an oncogene, it is not known whether it is associated with *TAOS1*-related tumorigenesis as in the case of *BYSL*.

Finally, we investigated whether 6p21 gain/amplification of DLBCL was reflected in the clinical data. Four cases with 6p21 gain could be subjected to gene expression clustering (19). These four cases were evenly distributed into activated B-cell-like and germinal center B-cell-like types. We found that 6p21 gain was frequently found in younger patients (<60 years, $P = 0.02$) but no significance was found for other prognostic factors, such as lactate dehydrogenase, performance status, and stage. Additional studies will be needed to confirm these observations in larger series of patients.

In summary, although it is known that t(6;14)(p21;q32) induces aberrant overexpression of *CCND3* in B-cell malignancies, we were able to show that *CCND3* can be also a target of genomic gain/amplification. Overexpression of *CCND3* through genomic gain/amplification is likely to lead to aberrant cell cycle control, although the precise biological role of *BYSL* with respect to tumorigenesis remains unknown. Further biological studies are needed to determine the tumorigenic function of these candidate genes in DLBCL.

Acknowledgments

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Post-transplant events

Solid tumors after hematopoietic stem cell transplantation in Japan: incidence, risk factors and prognosis

K Shimada, T Yokozawa, Y Atsuta, A Kohno, F Maruyama, K Yano, H Taji, K Kitaori, S Goto, H Iida, Y Morishima, Y Kodera, T Naoe and Y Morishita

Nagoya Blood and Marrow Transplantation Group (NBMTG), Nagoya, Japan

Summary:

To evaluate the incidence, risk factors and prognosis for solid tumors after hematopoietic stem cell transplantation (HSCT) in Japan, 809 patients who had received HSCT between 1981 and 2000 were retrospectively analyzed. In all, 19 newly diagnosed secondary cancers were observed. The risk for cancer development was 2.8 times as high as that for expected cases. The cumulative incidence ratios at 5 and 10 years were 1.9 and 4.2%, respectively. The risk was significantly elevated for buccal cavity cancer (standard incidental ratio (SIR), 44.42: 95% confidence interval (CI) 17.86–91.51), esophageal cancer (SIR, 22.36: 95% CI 6.09–57.25), and cervical cancer (SIR, 8.58: 95% CI 1.04–31.01). Of 15 patients who developed solid cancers following allografting, 12 had chronic graft-versus-host disease (GVHD), and all 10 patients with squamous cell carcinoma of the buccal cavity or esophagus had chronic GVHD. On multivariate analysis, extensive chronic GVHD and age over 45 years at the time of transplantation were associated with a higher risk for solid cancers. In all, 17 patients received therapy for secondary cancers, nine of whom are still alive and the 5-year probability of survival from the diagnosis is 42.8%. Our data suggest that early detection of secondary cancers is very important in prolonging overall survival.
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Hematopoietic stem cell transplantation (HSCT) is widely used to treat patients with hematologic malignancies and other nonmalignant hematologic disorders. The improvement in survival after HSCT has drawn increased attention to late complications in long-term survivors. The development of second malignancies is recognized as one of the most serious complications in such patients.[1] Second malignancies following HSCT fall into three categories: solid tumors, therapy-related myelodysplastic syndrome/acute myeloid leukemia (t-MDS/AML), and post-transplant lymphoproliferative disorder.[2–5] For solid tumors, the incidence in previous reports was found to be 2–6% at 10 years, and 6–13% at 15 years.[1,6–8] Although these studies have demonstrated a low but significant risk of a second solid tumor after HSCT, few data exist in Japanese patients with different specific incidence rates for cancers at specific anatomical sites. We undertook a retrospective analysis of 809 Japanese patients who received HSCT, using the Nagoya Blood and Marrow Transplantation Group (NBMTG) database to assess the incidence and risk factor of solid tumors following HSCT. In addition, we present the treatment outcomes of these patients.

Patients and methods

Patients and treatment

A total of 1451 patients who received allogeneic and autologous HSCT at 16 institutes cooperating in the NBMTG between 1981 and 2000 were identified from the computerized NBMTG database, where pretransplant and post-transplant data are consecutively collected and maintained on a regular basis by requesting follow-up information and reporting of events. For each second cancer identified in the database, clinical data including date of diagnosis, type and characteristics of the cancer, method of treatment, and outcome were obtained. Each record and pathology report was reviewed to ensure accuracy of the database, contents, and updated for the current analysis.

Patient characteristics are detailed in Table 1. Among the 809 patients who survived for at least 1 year after transplantation, the median age at transplantation was 34 years (range, 15–70 years). Allogeneic transplantation was performed in 591 patients, 211 patients received autologous marrow and seven patients received syngeneic donor marrow. In all, 410 patients received donations from an HLA-identical sibling and 31 from

Correspondence: Dr K Shimada, Division of Hematology and Oncology, JA Aichi Showa Hospital, 46 Nobaku, Nobaku-cho, Konan, Aichi 483-8703, Japan; E-mail: kzshima@fan.hi-ho.ne.jp
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Table 1 Characteristics of patient population

	Total		*Solid tumors*
	Number	*%*	*Number*
No. of patients	809		19
Sex			
Male	485	60.0	10
Female	324	40.0	9
Age at HSCT (years)			
Range	15–70		28–61
Median	34		46
Primary diagnosis			
AA	57	7.0	0
AML	153	18.9	4
ALL	139	17.2	3
NHL	135	16.7	4
HL	15	1.9	1
CML	186	23.0	4
MDS	42	5.2	1
MM	18	2.2	1
ATL	3	0.4	0
Breast cancer	38	4.7	1
Others	23	2.8	0
Type of HSCT			
Allogeneic	591	73.1	12
Sibling	410	50.7	12
Other relative	31	3.8	0
Unrelated	150	18.5	3
Syngeneic	7	0.9	0
Autologous	211	26.1	4
Conditioning regimen			
TBI	475	58.7	8
Non-TBI	325	40.2	11
GVHD prophylaxis			
MTX + CSP	489	60.4	14
Others	99	7.9	1
None	214	26.5	4
Acute GVHD total	343	42.4	9
Grades II to IV	106	13.1	5
Chronic GVHD total	263	32.5	12
Limited type	106	13.1	3
Extensive type	157	19.4	9

HSCT = hematopoietic stem cell transplantation; TBI = total body irradiation; MTX = methotrexate; CSP = cyclosporine.

other family donors. A total of 150 patients received unrelated donor marrow grafts matched for HLA phenotype. Most of the grafts for allogeneic transplantation were bone marrow. Conditioning regimens for 475 patients consisted of total body irradiation (TBI) combined with cyclophosphamide and/or other drugs. Prophylaxis for graft-versus-host disease (GVHD) consisted mainly of methotrexate plus cyclosporine in 489 patients (82.7% of allogeneic transplants). Acute GVHD (grade II–IV) developed in 106, and chronic GVHD in 263 patients (17.9 and 44.5% of allogeneic transplants), respectively. The median duration of follow-up was 5.3 years (range, 1.0–19.9 years) and 6.6 years for survivors.

Statistical analysis

Standardized incidence ratios (SIRs) were calculated to determine whether the number of patients in our cohort who developed a second cancer after receiving a transplant was excessive. These calculations are based on the ratio of the number of patients who developed subsequent invasive cancers after their transplant (observed number) to the number of people in the general population who would be expected to develop primary invasive cancer (expected number). The latter number was determined as follows: for each patient, the number of person-years at risk was calculated from the date of transplantation until the date of the diagnosis of second cancer, date of the last contact, or death, whichever came first. Age (5 years)-, sex-, and calendar year (5 years)-specific incidence rates for all cancers at specific anatomical sites were applied to the appropriate person-years at risk to compute the expected numbers of cancers. The median year of each 5-year period was chosen to represent the incidence rate of that period. For the last period (from 2000 to 2003), the most recent incidence-rate data available (from the year 1998) were used. Incidence rates for all invasive cancers were obtained from the data base of the Research Group for Population-based Cancer Registration in Japan.[9–12] The 95% confidence intervals (CIs) for the SIRs were calculated on the assumption that the observed number of cancers followed a Poisson distribution.[13] Cumulative probabilities for the development of secondary solid tumors were calculated by the Kaplan–Meier method with 95% CI calculated using Greenwood's formula. The log-rank test was used to compare the distributions between groups. The influence of potential risk factors was estimated by the Cox proportional-hazard model. Variables selected were those previously described. A stepwise multivariate approach was used to identify the most important predictor variable with respect to the development of secondary solid tumors. A P-value <0.05, after adjustment for the effects of other variables, was required for inclusion in the model. The Kaplan–Meier method was used to calculate survival probability among patients who developed solid tumors.

Results

Among the 809 patients who underwent stem cell transplantation and survived more than 1 year, 19 new cases of solid cancer were identified (Table 2). Seven patients were diagnosed with buccal cavity carcinoma, and the remaining 12 with secondary cancers included three with esophageal, two each with cervix uteri and gastric cancer, and one each with uterine cancer, rectal cancer, colon cancer, breast cancer, and squamous cell carcinoma of unknown origin. The latency period from HSCT to the emergence of a new solid cancer ranged from 12 to 139 months. The cumulative incidence rates at 5 and 10 years after transplantation were 1.9 and 4.2%, respectively (Figure 1). The overall risk of developing new solid cancers had significantly increased, with 19 invasive cancers observed compared with 6.7 cases expected in an age- and sex-matched general population (standard incidental ratio

Table 2 Patient characteristics of secondary malignancies

No.	Age (years)	Sex	Secondary cancers	Primary diseases	Donor relation	Pre- and post-transplant RT	Conditioning regimen	TBI	aGVHD (II–IV)	cGVHD	IM at diagnosis	Latency from HSCT (mo)	Therapy	Outcome	Survival from Dx (mo)
1	39	M	Buccal Ca	ALL	Sibling	N	CY	12	Y	EX	Y	84	ST	Alive	115^{+}
2	49	F	Uterus Ca	Breast Ca	Auto	Y	CY, E, Thio	0	N	N	N	84	ST + CT	Alive	79^{+}
3	30	M	Buccal Ca	CML	Unrelated	N	CA, CY	12	N	EX	Y	36	ST + RT	Alive	99^{+}
4	47	F	Buccal Ca	CML	Sibling	N	BU, CY	0	N	EX	Y	68	ST	Alive	78^{+}
5	29	M	Gastric Ca	HL	Auto	Y	E, CY, Mel	0	N	N	N	63	ST	Dead	43
6	55	M	Rectum Ca	AML	Sibling	N	BU, CY	0	N	LD	N	69	ST	Alive	47^{+}
7	61	M	Esophageal Ca	AML	Sibling	N	BU, CY	0	N	EX	N	59	CT + RT	Dead	23
8	46	F	Buccal Ca	CML	Sibling	N	BU, CY	0	Y	EX	Y	39	CT + RT	Dead	3
9	44	F	Esophageal Ca	AML	Sibling	N	BU, CY	0	N	EX	Y	74	CT + RT	Dead	8
10	28	F	Cervical Ca	ALL	Unrelated	N	CA, CY	12	N	N	N	46	ST + CT	Dead	34
11	44	F	Gastric Ca	MM	Sibling	N	Mel	12	N	EX	N	53	BSC	Dead	3
12	30	F	Cervical Ca	CML	Sibling	N	CA, CY	12	Y	N	N	59	ST	Alive	40^{+}
13	55	M	Buccal Ca	ALL	Sibling	Y	BU, CY	0	N	LD	N	41	CT + RT	Dead	34
14	61	M	Colon Ca	NHL	Auto	N	CY, Mel, E, Dex	0	N	N	N	27	ST	Alive	39^{+}
15	51	F	Breast Ca	MDS	Unrelated	N	BU, CY	10	N	N	Y	12	ST	Alive	39^{+}
16	49	M	SCC (UO)	NHL	Auto	N	E, CBDCA, Mel	0	N	N	N	74	RT	Dead	9
17	34	M	Buccal Ca	NHL	Sibling	Y	Mel, TAI	0	N	EX	Y	73	ST	Alive	12^{+}
18	29	M	Buccal Ca	NHL	Sibling	Y	E, Mel	12	Y	LD	N	139	BSC	Dead	3
19	48	F	Esophageal Ca	AML	Sibling	N	BU, CY	12	Y	EX	N	43	ST	Dead	5

Ca = cancer; CY = cyclophosphamide; E = etoposide; Thio = thiotepa; BU = busulfan; Mel = melphalan; Dex = dexamethasone; TAI = thoracoabdominal irradiation; IM = immunosuppression; aGVHD = acute graft-versus-host disease; cGVHD = chronic graft-versus-host disease; EX = extensive type; LD = limited type; mo = months; SCC = squamous cell carcinoma; UO = unknown origin; ST = surgical therapy; CT = chemotherapy; RT = radiation therapy; BSC = best supportive care; Dx = diagnosis of secondary cancers; M = male; F = female; Y = yes; N = no.

(SIR), 2.82: 95% CI 1.70–4.40), and specifically for cancers of the buccal cavity (SIR, 44.42: 95% CI 17.86–91.51), esophageal cancer (SIR, 22.36: 95% CI 6.09–57.25), and cervical cancer (SIR, 8.58: 95% CI 1.04–31.01).[9–12] On the other hand, the risk of gastric cancer, which is the most common cancer in Japan, had not increased compared with that in the general population (SIR, 1.37: 95% CI 0.17–4.95) (Table 3).

As shown in Table 2, 12 patients had undergone allogeneic transplantation from an HLA-matched sibling, three from an unrelated donor, and four had received autologous marrow. Eight patients received TBI-containing preparative regimens before allogeneic transplant. Remarkably, 12 of 15 allo-grafted patients had developed chronic GVHD, nine of whom had the extensive type. Six patients of those nine were receiving immunosuppressive agents at the time of diagnosis of the new cancer. Squamous cell carcinoma of the buccal cavity or esophagus originated from a site involved with chronic GVHD. Univariate regression analyses were performed for the patients who survived for at least 1 year after transplantation. Potential risk factors were evaluated with adjustments for age, sex, type of transplantation, donor, chronic GVHD, etc. Patients with extensive chronic GVHD aged over 45 years had an elevated risk of all solid cancers. Relative risk (RR) was 2.9 (95% CI 1.1–7.2) and 5.1 (95% CI 2.0–12.6), respectively. On multivariate analysis, age, sex, TBI, pretransplant radiotherapy, acute GVHD, and chronic extensive GVHD were added to the Cox proportional-hazard model. Two independent risk factors, that is, extensive chronic GVHD (RR = 2.9: 95% CI 1.1–7.8, $P = 0.0352$) and age over 45 years (RR = 5.5: 95% CI 1.9–13.8, $P = 0.0011$), were identified.

Of the 19 patients with new cancers, 17 received various therapies for secondary cancers (Table 2). Surgery was performed on 12 patients, of which eight operations were curative. All of those who received a curative operation survived more than a year from diagnosis (range 12–115 months). Only one out of three patients treated with chemotherapy and radiotherapy survived about 3 years from diagnosis. Nine out of 19 patients are still alive with no signs of tumor regrowth. The remaining 10 have died from secondary cancers. Finally, the probability of a 5-year survival of patients from their diagnosis of secondary cancers is 42.8% (Figure 2).

Discussion

Among the 809 adult patients who received HSCT by the Nagoya Blood and Marrow Transplantation Group

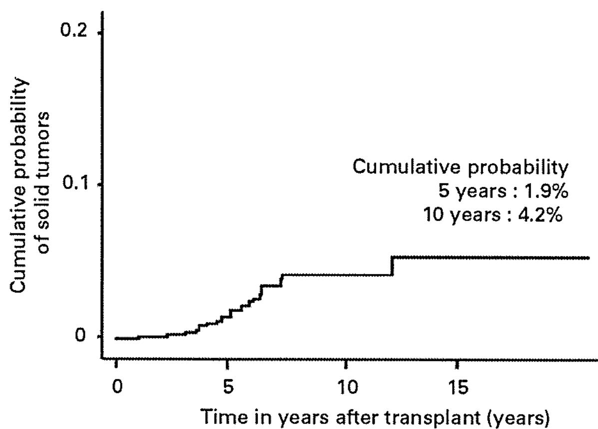

Figure 1 Cumulative probability of solid cancers after HSCT. The probabilities at 5 and 10 years were 1.9 and 4.2%, respectively.

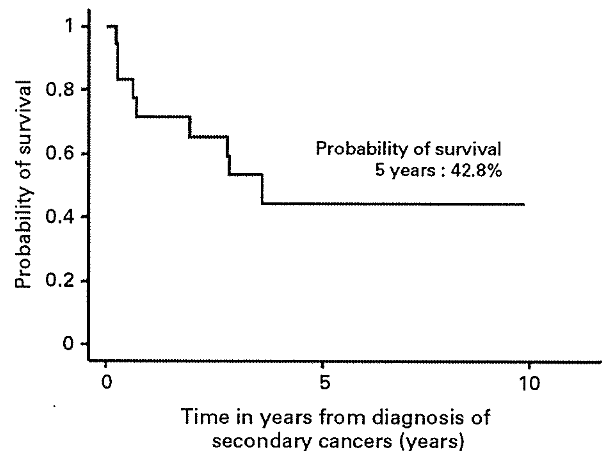

Figure 2 Kaplan–Meier survival for patients diagnosed with secondary cancers. The 5-year probability of survival is 42.8%.

Table 3 SIRs of invasive subsequent cancers

No. of patients (person-year)		809 (4704.2)		
Site of cancer	*Obs*	*Exp*	*SIR (Obs:Exp)*	*95% CI (SIR)*
All solid tumors	19	6.74	2.82	1.70–4.40
Oral or pharynx	7	0.16	44.42	17.86–91.51
Esophagus	4	0.18	22.36	6.09–57.25
Stomach	2	1.46	1.37	0.17–4.95
Colon	1	0.72	1.39	0.04–7.77
Rectum	1	0.49	2.05	0.05–11.43
Breast	1	1.06	0.95	0.02–5.28
Cervix	2	0.23	8.58	1.04–31.01
Uterine corpus	1	0.12	8.5	0.22–47.38

Obs = observed number; Exp = expected number; CI = confidence interval.

between 1981 and 2000 and who survived for more than 1 year after transplant, 19 have developed a new solid cancer with an estimated average cumulative risk of a new solid cancer of 1.9% at 5 years and 4.2% at 10 years after transplantation. The overall incidence is 2.8-fold higher than that expected in the general population. This analysis represents one of the largest surveys in Japan. In our cohort of patients, secondary hematological malignancies such as Epstein–Barr virus-associated lymphoma, leukemia, and MDS were excluded, since the spectrum of neoplasms varies with the time after transplantation.[4,14,15] Hematologic malignancies generally develop in the early phase following transplantation.[5] On the other hand, solid cancers have a longer latency period.[1,6] Therefore, this figure certainly represents a significant risk to long-term survival among adult patients undergoing HSCT. The RR obtained from our analysis could be an underestimate of the true risk since the median follow-up duration of 5.3 years is relatively short. In contrast to the other studies, the cumulative incidence of developing a solid cancer plateaued with a relatively short latency. Although this is mainly a consequence of the short follow-up and of the fact that the peak incidence of solid cancers had not been reached, the difference in the spectrum and pathogenesis of secondary cancer may also be a factor.

Potential risk factors associated with the development of secondary cancers following HSCT have been described previously.[1,6,16–18] These include TBI as part of the preconditioning regimen, the use of immunosuppressive agents, and the occurrence of acute or chronic GVHD. In our study, two independent risk factors were identified, namely, subjects with extensive-type chronic GVHD and those above 45 years of age at transplant. TBI containing preconditioning was not a statistically significant risk factor. Radiogenic cancers generally have a long latent period, and the risk of such cancers is frequently high among patients undergoing irradiation at a young age.[17,19] Certain tumor types such as those of the brain, thyroid, salivary gland, and bone connective tissue occur in association with radiation exposure.[20–24] These tumors were not observed in our study, which included only adult patients. In two large studies, brain and thyroid cancers accounted for the increased risk of TBI in their cohorts of children.[1,6] The risk of radiogenic cancers may decline with age. According to the previous report, the cumulative incidence of second cancer patients who had received TBI was similar for younger and older patients.[25] However, the difference between patients with and without TBI exposure was not evident among those more than 10 years of age.

A recent large study concerning chronic GVHD and its therapy reported the risk of solid cancers, particularly squamous cell carcinomas of the buccal cavity and skin.[26] In our study, the most significant risk for specific types of secondary solid tumors was to patients with squamous cell carcinoma of the oral cavity and esophagus, which in this study was found to be 44- and 22-fold higher than that expected in the general population, respectively. All 10 patients with these cancers had chronic GVHD, eight of whom had the extensive type. Although the most common types of secondary malignancy are epithelial in origin,[1] cutaneous cancers were encountered most frequently among patients in Western countries. This discrepancy may be partly explained either by the difference in the clinical manifestation of chronic GVHD or by a genetic susceptibility and a gene–environment interaction. On the other hand, skin involvement of greater than 50% was apparent only in approximately 10% of Japanese patients with chronic GVHD (unpublished data by NBMTG), whereas in the data of Akpek *et al*,[27] 44% of patients presented with extensive skin involvement, indicating that the incidence of skin cancer is much lower among the Japanese population. Gastric cancer, on the other hand, is the most prevalent neoplasm in Japan, accounting for one-quarter of all cancers.[9–12] In our study, only two cases of gastric cancer were observed after HSCT, an incidence not statistically different from that in the general population. This indicates the existence of a distinctive mechanism underlying the development of post-transplant secondary cancers.

Patients with chronic GVHD, having received immunosuppressive agents over a long period, exhibit persistent inflammation in the involved organs. GVHD may stimulate regeneration of the epithelium and a subsequent overgrowth of malignant cells. Alternatively, prolonged immune suppression may compromise immune surveillance and exert a cocarcinogenic effect on the genetic damage caused by chemoradiation. Host defenses and inflammatory gene polymorphisms may be associated with an increased risk of GVHD,[28] and may also play a role together with DNA repair in solid tumors. In immunosuppressed patients, oncogenic viruses such as human papilloma viruses may contribute to squamous cell carcinoma of the cervix and skin.[29–32] In our study, two patients developed cervical cancer with an increased risk of 8.6 of RR.

The magnitude of the increased risk of solid tumors after autologous HSCT has been reported to be approximately two-fold when compared to that in an age- and sex-matched general population.[33] A 5-year cumulative incidence of 8.9% was reported for autologous HSCT patients treated for Hodgkin's disease, which was more frequent than that for conventionally treated patients.[34] Four patients developed secondary cancers after autologous transplantation in our study. However, no significant risk factor was detected for the autologous transplantation on multivariate analysis (data not shown). The predominance of cancers of epithelial origin was not observed in our autologous patients. The carcinogenic process may differ from that in the setting of allogeneic transplantation. The difference between autologous and allogeneic transplantation may include the type of disease for which both groups were transplanted, chemoradiotherapy prior to transplant and the presence or absence of an immunoresponse. In fact, about 60% of the allogeneic patients received transplantation for myeloid malignagncies, on the other hand, about 85% of autologous transplant patients had nonmyeloid malignancies.

Although the prognosis for secondary malignancies was generally poor,[35,36] there was one report of four of five patients who survived after undergoing the same therapy used for *de novo* cancers.[37] In another recent study, the 5-year survival rate of secondary cancers was 44%.[7] The

prognosis mostly depends on whether the secondary cancers can be diagnosed early enough to begin the standard therapy appropriate for each cancer. In our study, most of those who received curative care overcame their secondary cancers, resulting in an overall survival rate of 42.8% at 5 years. It is important for all physicians to be alert to the possibility of secondary malignancy after HSCT, and to monitor more closely patients who developed chronic GVHD.

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Comparison of genome profiles for identification of distinct subgroups of diffuse large B-cell lymphoma

Hiroyuki Tagawa, Miyuki Suguro, Shinobu Tsuzuki, Keitaro Matsuo, Sivasundaram Karnan, Koichi Ohshima, Masataka Okamoto, Yasuo Morishima, Shigeo Nakamura, and Masao Seto

Diffuse large B-cell lymphoma (DLBCL) comprises molecularly distinct subgroups such as activated B-cell-like (ABC) and germinal center B-cell-like (GCB) DLBCLs. We previously reported that CD5$^+$ and CD5$^-$CD10$^+$ DLBCL constitute clinically relevant subgroups. To determine whether these 2 subgroups are related to ABC and GCB DLBCLs, we analyzed the genomic imbalance of 99 cases (36 CD5$^+$, 19 CD5$^-$CD10$^+$, and 44 CD5$^-$CD10$^-$) using array-based comparative genomic hybridization (CGH). Forty-six of these cases (22 CD5$^+$, 7 CD5$^-$CD10$^+$, and 17 CD5$^-$CD10$^-$) were subsequently subjected to gene-expression profiling, resulting in their division into 28 ABC (19 CD5$^+$ and 9 CD5$^-$CD10$^-$) and 18 GCB (3 CD5$^+$, 7 CD5$^-$CD10$^+$, and 8 CD5$^-$CD10$^-$) types. A comparison of genome profiles of distinct subgroups of DLBCL demonstrated that (1) ABC DLBCL is characterized by gain of 3q, 18q, and 19q and loss of 6q and 9p21, and GCB DLBCL is characterized by gain of 1q, 2p, 7q, and 12q; (2) the genomic imbalances characteristic of the CD5$^+$ and CD5$^-$CD10$^+$ groups were similar to those of the ABC and GCB types, respectively. These findings suggest that CD5$^+$ and CD5$^-$CD10$^+$ subgroups are included, respectively, in the ABC and GCB types. Finally, when searching for genomic imbalances that affect patients' prognosis, we found that 9p21 loss (*p16^{INK4a}* locus) marks the most aggressive type of DLBCL. (Blood. 2005; 106:1770-1777)

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Introduction

Diffuse large B-cell lymphoma (DLBCL) is the most common type of non-Hodgkin lymphoma and is known to include pathophysiologically heterogeneous groups.[1-4] DLBCL is also known to be clinically heterogeneous because patients with DLBCL show markedly different clinical courses.[5] This has drawn attention to the importance of identifying subgroups in heterogeneous DLBCL.

Gene-expression profiling conducted by Alizadeh et al[6] identified 2 molecularly distinct forms of DLBCL with gene expression patterns indicative of the different stages of B-cell differentiation, that is, activated B cell-like (ABC) and germinal center B-cell-like (GCB) types.[6,7] The ABC group expresses genes characteristic of activated B cells and plasma cells, whereas the GCB group maintains the gene expression program of normal germinal center B cells.[6-8] Those authors also reported that the overall survival of the ABC group was significantly worse than that of the GCB group.

We reported the identification of 3 phenotypically distinct subgroups of DLBCL, CD5$^+$, CD5$^-$CD10$^+$, and CD5$^-$CD10$^-$ DLBCLs.[9] The CD5$^+$ group, found to account for approximately 10% of all DLBCL, has the CD5$^+$C10^{+-}CD19$^+$CD20$^-$CD21$^-$CD23$^-$cyclinD1$^-$ phenotype and is characterized by poorer prognosis, frequent extranodal sites, poorer performance status, and higher lactase dehydrogenase levels compared with CD5$^-$ DLBCL.[10] The CD5$^-$CD10$^+$ group shows less-frequent BCL2 protein expression than the other groups, indicating the presence of a definite relationship with normal germinal center cells that usually lack BCL2 expression. Finally, the CD5$^-$CD10$^-$ group is the most common group and has a higher incidence of *BCL6* gene rearrangement than the other 2 groups, although the difference is insignificant.[9]

Despite each subgroup of DLBCL being molecularly or phenotypically distinct, the genetic characteristics and their relationship have not been sufficiently studied. Here, we made use of the array-based comparative genomic hybridization (array CGH) to identify genomic imbalances characteristic of the distinct subgroups of DLBCL.[11,12] Gene-expression profiling was also used to clarify the relationship between the ABC/GCB and CD5$^+$/CD5$^-$CD10$^+$ subgroups.

Patients, materials, and methods

Patients and samples

Lymph node samples and clinical data were obtained from 99 patients with the protocol approved by the Institutional Review Board of Aichi Cancer Center (36 cases of CD5$^+$, 19 cases of CD5$^-$CD10$^+$, and 44

From the Division of Molecular Medicine, Aichi Cancer Center Research Institute, Nagoya, Japan; the Japan Biological Informatics Consortium, Tokyo, Japan; the Division of Epidemiology and Prevention, Aichi Cancer Center Research Institute, Nagoya, Japan; the First Department of Pathology, Fukuoka University School of Medicine, Fukuoka, Japan; the Department of Internal Medicine, Fujita Health University School of Medicine, Toyoake, Aichi, Japan; the Department of Hematology and Cell Therapy, Aichi Cancer Center Hospital, Nagoya, Aichi, Japan; and the Department of Pathology and Molecular Diagnostics, Aichi Cancer Center Hospital, Nagoya, Aichi, Japan.

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Reprints: Masao Seto, Division of Molecular Medicine, Aichi Cancer Center Research Institute, 1-1 Kanokoden, Chikusa-ku, Nagoya 464-8681, Japan; e-mail: mseto@aichi-cc.jp.

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