Table 2 Frequency of the mutation (c.3175G>T) in various tissues

Tissue	Subject	Frequency (%)	No of reads		
			Total	Ref allele (G)	Variant allele (T)
Blood(control)	1	0.0	64489	31023+ 33386−	6+ 0−
Blood	1	12.2	3726	1705+ 1566−	302+ 151−
Hair follicles	1	4.5	83679	39527+ 40249−	1918+ 1856−
Buccal mucosa	1	3.0	69169	32605+ 34368−	1047+ 1052−
	2	7.3	66557	30510+ 31082−	2477+ 2383−
Gastric mucosa	1	18.9	81041	29167+ 36446−	7025+ 8228−
	2	22.7	74101	27245+ 29871−	8089+ 8654−
	3	27.7	84151	27281+ 33392−	11009+ 12284−
Normal coloic mucosa	1	9.2	5149	2419+ 2252−	290+ 182−
	2	3.4	4698	2248+ 2291−	83+ 76−
	3	12.3	4321	1978+ 1807−	327+ 206−
	4	5.8	4514	2226+ 2026−	165+ 96−
	5	9.0	4097	2123+ 1602−	267+ 103−
Polyp	1	32.3	4797	1687+ 1557−	1013+ 536−
	2	28.6	4543	1883+ 1356−	937+ 362−
	3	29.8	4402	1537+ 1551−	841+ 469−
	4	32.5	4554	1628+ 1442−	981+ 494−
	5	24.7	4166	1581+ 1549−	685+ 344−

+,− indicate strand reads.

polyposis phenotype than the expected phenotype based on the site of the mutation.[4,6] In agreement with this notion, our case showed an attenuated form of polyposis demonstrating less than 100 polyps in the intestine, although the mutation of *APC* c.3175G>T, p.E1059X was reported to exhibit a florid form of adenomatous polyps in the large intestine at a young age and additional extra-colonic manifestations (duodenal adenoma and fundic gland polyps) in a FAP patient.[18] Application of NGS in genetic testing for patients with polyposis may increase the frequency of *APC* mosaicism in cases without familial history and those with mild phenotype.

After the surveillance of large intestine for eight years, the patient underwent subtotal colectomy in combination with ileorectal anastomosis because two of four biopsies of the colonic polyps histologically showed severe atypia. As we were unable to examine the DNA of his parents, we could not confirm *de novo APC* mutation in the patient. In addition, although his children have not been investigated, we should consider the possibility that germline mosaicism may lead to more severe phenotypes in the next generation.

On the basis of our result from deep sequencing, the frequency of the mutant allele was not constant among different types of tissues, and even in different sections isolated from the same tissue. The mutant allele was also found to a relatively higher extent in normal gastric mucosa (18.9–27.7%) compared with that in peripheral blood (12.2%), hair follicles (4.5%), buccal mucosa (3.0–7.3%) and normal colonic mucosa (3.4–12.3%). During embryogenesis, a zygote starts development and forms three germ layers; ectoderm, endoderm and mesoderm. Peripheral blood originates from mesoderm, hair follicles and buccal mucosa from ectoderm, and gastric and colonic mucosa from endoderm. Therefore the mutation should occur before the separation of these three layers. Although cells carrying the mutation were delivered into different tissues at different frequencies, we may assume from the frequencies that the mutation occurred at the four- or eight-cell stage. As the patient carried the mutation in non-tumorous gastric mucosa at a relatively high frequency, future surveillance paying special attention to his stomach is essential because the relative risk for gastric cancer in FAP patients is higher than the normal population in Asia.[19,20] Interestingly we also found that the mutation frequency is different at the location even in the same tissue, suggesting a possibility of intra-organic mosaicism.

Sequencing by the Sanger method is a gold standard of genetic testing for FAP. However, it is reported that the sensitivity of mutation by the direct sequence method is ~15%.[21] Consistent with this data, we failed to identify the mutation in our initial screening. Although PTT, DGGE, DHPLC and HRM are also applicable for the initial screening and may have higher sensitivities compared with Sanger's method, they are indirect detection systems and additional confirmatory sequencing is essential. In the near future, sequencing by NGS will replace the screening strategies for polyposis because the cost for NGS is dramatically decreasing. Previous reports also revealed the effectiveness of NGS for the detection of mosaic mutations.[21,22] Consistently, our data show that amplicon sequence with NGS is useful for the quantification of mosaic mutation (Figure 1 and Table 2). We additionally confirmed the mutation using a different set of primers, and the degree of mosaicism was comparable to that using the initial set of primers (data not shown), suggesting that the ratio is not affected by the set of primers used. Therefore, the amplicon-based NGS approach is reliable for confirming low-level mutation and quantifying mosaic mutations. It is of note that the sensitivity of detection of mosaic mutation by NGS totally depends on the number of reads. Although we identified the mosaic mutation in 6 out of 50 reads, we might miss the mutation if the depth of reads at the region was less than 10 or so. Although the sensitivity to identify low levels of mosaic mutation will be increased by the use a high depth sequence method such as amplicon sequence or whole-exome sequence, these methods may overlook structural changes such as translocation and deletion/amplification of large regions. Therefore, we should take advantage of the most appropriate method for the detection of different types of alterations. In addition, it may be possible to analyze multiple polyps, if available, in the patients, because the tumors are largely composed of relatively homogeneous cell population, and the mosaic mutation should be shared in the DNA from the polyps.

In conclusion, we successfully identified a mosaic mutation in a patient with a fraction of 12% mutated allele in peripheral blood by whole-genome sequencing covered at 26x average depth. As mutational mosaicism of the *APC* gene has relevance to cancer risk, genetic diagnosis is useful for the decision of surveillance and personalized treatment of the patients, and may be applied for the pre-symptomatic diagnosis of their children. These results will accelerate the application of NGS in clinical settings.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Identification of Two Wnt-Responsive Elements in the Intron of RING Finger Protein 43 (RNF43) Gene

Norihiko Takahashi, Kiyoshi Yamaguchi, Tsuneo Ikenoue, Tomoaki Fujii, Yoichi Furukawa*

Division of Clinical Genome Research, Advanced Clinical Research Center, Institute of Medical Science, The University of Tokyo, Tokyo, Japan

Abstract

RING finger protein 43 (RNF43), an E3-type ubiquitin ligase, is frequently up-regulated in human colorectal cancer. It has been shown that expression of *RNF43* is regulated by the Wnt-signaling pathway. However the regulatory region(s) for its transcriptional activation has not been clarified. In this study, we have shown for the first time that *RNF43* is a direct target of TCF4/β-catenin complex, and that its expression is regulated by a regulatory region containing two Wnt-responsive elements (WREs) in intron2. A reporter gene assay revealed that nucleotide substitutions in the WREs decreased the reporter activity in colon cancer cells, suggesting that both WREs are involved in the transcriptional activation. Knockdown of β-catenin by siRNA suppressed the reporter activity. In addition, ChIP assay showed that both elements associate with TCF4/β-catenin complex in colon cancer cells. These data indicate that expression of *RNF43* is regulated by the canonical Wnt/β-catenin pathway through binding of the WREs with TCF4/β-catenin complex. These findings should be useful for the understanding of the regulatory mechanism of RNF43 and may contribute to the clarification of signaling pathways regulated by RNF43.

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* E-mail: furukawa@ims.u-tokyo.ac.jp

Introduction

Colorectal cancer is one of the most common malignancies worldwide, and the third most common cancer-related death in Japan and in the United States of America. In the US, it is estimated that 142,820 of new cases will be diagnosed and that 50,830 patients will die of this disease in 2013 (SEER stat fact sheets, http://seer.cancer.gov/statfacts/html/colorect.html) [1]. Although tumors at early stages are cured by surgery, those with far advanced stages are not curable by operation alone. Molecular targeted drugs, such as bevacizumab, cetuximab, and panitumumab, have been approved for the combination therapies to advanced colorectal cancer, and have improved the efficacy of chemotherapies. Nevertheless, the five-year survival rate of metastatic cancer is still lower than 12% [1], suggesting that novel therapeutic strategies are needed.

Molecular studies have clarified that deregulation of the Wnt signaling pathway is involved in colorectal carcinogenesis. Wnt signal regulates differentiation, proliferation, compartmentation, and cell fate of epithelial cells in the intestinal mucosa. One of the key mediators of the pathway is β-catenin, which also plays a structural role in cell-cell adhesion by binding to cadherins [2]. In the absence of Wnt signaling, a multi-molecular complex comprising of β-catenin, APC, Axin/Axin2 (or Conductin) and glycogen synthase kinase 3β (GSK3β) phosphorylates β-catenin, leading to its ubiquitination and subsequent degradation in the proteosome [3]. Upon binding with the Frizzled family and LRP receptor complexes, Wnt proteins activate Dishevelled (Dvl) proteins that inhibit activity of glycogen synthase kinase 3β [4]. As a result, degradation of β-catenin is suppressed and accumulated β-catenin induces TCF/LEF-mediated transcription [5,6].

In colorectal cancer cells, frequent mutations are observed in *APC*, the responsible gene for familial adenomatous polyposis of the colon, and *β-catenin (CTNNB1)* [4,7]. In hepatocellular carcinomas, frequent mutations are found in *CTNNB1* and *AXIN1* [4,8,9]. These mutations are mutually exclusive, and result in transactivation of TCF/LEFs, members of high mobility group (HMG) box protein family [5,9], suggesting that mutation in one of these components is enough to abrogate canonical Wnt signaling, and that TCF/LEF mediated transcriptional activation is important for these tumors. It has been thought that TCF4/β-catenin complex bend the DNA to access distant DNA region and form correct chromatin conformations for efficient RNA polymerase II (pol II)-mediated transcription [10]. Consequently, downstream target genes such as *c-myc* [11], *cyclinD1* [12], *MMP-7 (matrilysin)* [13,14], *urokinase-type plasminogen activator receptor (uPAR)* [15], *connexin 43* [16], *CD44* [17], *PPAR-delta* [18], *AF17* [19], *ENC1* [20], *Laminin-5 γ2* [21], *Claudin-1* [22] and *MT1-MMP* [23] are activated.

Earlier assigned as a hypothetical protein FLJ20315, RNF43 was shown to be an ubiquitin E3 ligase that associates with a nuclear protein, HAP95 [24]. Recently, two groups revealed that RNF43 enhances degradation of Wnt receptors including frizzled. One group showed that the degradation is mediated by the interaction with R-spondin proteins [25], and the other reported that this is carried out by endocytosis in LGR5-positive stem cells

in the intestine [26]. Interestingly, *RNF43* mutations were identified in a subset of pancreatic cancer [27,28], cholangiocarcinoma [29], colorectal cancer [30], and mucinous ovarian cancer [31]. These findings suggested that RNF43 is an important regulator of Wnt/β-catenin as well as Wnt/PCP pathway. In our earlier study, we found that expression of *RNF43* was frequently enhanced in colorectal cancer as well as hepatocellular carcinomas [32,33]. In addition, other groups revealed that *RNF43* expression was also elevated in adenomas of the colon [34], that it is down-regulated by a dominant-negative form of Tcf4 in LS174 colon cancer cells [35], and that expression of *RNF43* was induced by Wnt3a conditioned media [25]. These data suggested that *RNF43* is a downstream gene regulated by the Wnt-signaling pathway, but none has clarified the regulatory regions of its expression. In this study, we identified two Wnt-responsive elements (WREs) in intron2 of *RNF43* and found that these WREs are crucial for its transcriptional regulation through interaction with Tcf4/β-catenin complex. This is the first report of *RNF43* as a direct target of Tcf4/β-catenin complex and our data may be useful to understand the precise mechanism of RNF43 regulation.

Materials and Methods

Cell Lines

Human colorectal cancer cell lines, HCT116 and SW480, were obtained from the American Type Culture Collection (Manassas, VA). HCT116 cells were cultured in McCoy's 5A medium containing 10% fetal bovine serum (FBS, Life Technologies, Carlsbad, CA) and antibiotic/antimycotic solution (Sigma, St. Louis, MO). SW480 cells were cultured in Leibovitz's L-15 medium containing 10% FBS and antibiotic/antimycotic solution.

Gene Silencing

Human *CTNNB1*-specific siRNA were purchased from Dharmacon (ON-TARGETplus SMARTpool siRNA, L-003482-00). ON-TARGETplus Non-targeting Pool (D-001810-10) was used as a control. HCT116 or SW480 were seeded a day before treatment of siRNA. Cells were transfected with 15 nM of *CTNNB1*-specific or control siRNA using Lipofectamine RNAiMAX (Life Technologies). After 48 hours incubation, total RNAs were isolated with miRNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacture's instruction. The silencing effect was evaluated by quantitative RT-PCR and western blotting. Complementally DNA was synthesized from 1 μg of total RNA with Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics GmbH, Mannheim Germany). Real-time PCR was performed using SYBR Green technology with sets of primers (*RNF43*: forward primer, 5′-GTTTGCTGGTGTTGCTGAAA-3′, reverse primer, 5′-TGGCATTGCACAGGTACAG-3′, *GAPDH*: forward primer, 5′-AGCCACATCGCTCAGACA-3′, reverse primer, 5′-GCCCAATACGACCAAATCC-3′) for *RNF43* on StepOnePlus (Life Technologies). Amounts of transcripts were determined by relative standard curve method, and *GAPDH* was used as internal control.

Preparation of Reporter Plasmids

Putative promoter regions in the 5′-flanking region of RNF43 were amplified by PCR with two sets of primers (forward primer, 5′-AAAACGCGTCTACAGGGGAAACAATGTTGAAGGT-CAATAGGCT-3′, and reverse primer, 5′-AAACTCGAGTGGCCAGGTTTCTAGGCCCACTGC-3′ or 5′-AAACTCGAGTGGCAAAGAGAATGC-CAACTGGTGCTGT-3′, containing a recognition site of *Mlu*I or *Xho*I restriction enzyme (underlined nucleotides). PCR products were digested with the restriction enzymes and cloned into the appropriate enzyme sites of pGL3-Basic vector (Promega, Madison, WI). In addition, putative intronic enhancer region was amplified by PCR with sets of primers (fragment 1+2; 5′-AAAACGCGTAGACTATTTGGCTGTCTCAAAGT-CATTGCC-3′ and 5′-AAACTCGAGCCAGGGCCCAG-CATTGTGCCT-3′, fragment 1; 5′-AAAACGCGTAGAC-TATTTGGCTGTCTCAAAGTCATTGCC-3′ and 5′-AAACTCGAGTGGGGCATAGGCCCTGGTG-3′, fragment 2; 5′-AAAACGCGTCACCAGGGCCTATGCCCCAC-3′ and 5′-AAACTCGAGCCAGGGCCCAGCATTGTGCCT-3′, containing a recognition site of *Mlu*I or *Xho*I restriction enzyme (underlined nucleotides). PCR products were digested with the restriction enzymes, and cloned into the appropriate enzyme sites of pGL3-Promoter vector (Promega). Site-directed mutagenesis was carried out for both putative TCF4 binding sites, replacing CTTTGWW by CTTTGGC with the QuickChange II XL Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA) according to the manufacture's instruction.

Luciferase Assay

HCT116 or SW480 cells seeded on six-well plates were transfected with 1 μg of reporter plasmid and 0.1 μg of pRL-TK plasmid (Promega) by FuGENE 6 reagent (Roche) and incubated for 12 hours. Then the cells were further transfected with *CTNNB1* siRNA or control siRNA (ON-TARGETplus Non-targeting Pool #D-001810-10) at the concentration of 15 nM and incubated for an additional 36 hours. The cells were harvested and luciferase activities were measured using dual luciferase assay system (TOYO B-Net, Tokyo, Japan).

Chromatin Immunoprecipitation (ChIP) Assay

A ChIP assay was performed according to the Agilent Mammalian ChIP protocol with slight modifications. HCT116 or SW480 cells were cross-linked with 1% formaldehyde for 10 minutes at room temperature and quenched with 0.4 M glycine. Chromatin extracts were sheared by Micrococcal nuclease digestion, and subsequently protein-DNA complexes were immunoprecipitated with 3 μg of anti-TCF4 monoclonal antibody (6H5-3, Upstate, Charlottesville, VA) or anti-β-catenin monoclonal antibody (14/β-catenin, BD Transduction Laboratories, Franklin Lakes, NJ) bound to anti-mouse IgG-coated Dynabeads (Life Technologies). Non-immune mouse IgG (Santa Cruz Biotechnology, Santa Cruz, CA) was used as a negative control. The precipitated protein-DNA complexes were purified with the conventional DNA extraction method, and the DNAs were subjected to quantitative PCR analysis with the following primer sets; RNF43-int2-5′, forward primer, 5′-TCAACTCTCTGGA-TAAGGTGGAATAGC-3′, and reverse primer, 5′-GACTTTTGGGGTGGGTGGGAAATA-3′; RNF43-int2-3′, forward primer, 5′- TCGGGCACCTGGCCAAGATACA-3′, and reverse primer, 5′- TGGACGCCCTGGCTTCTGAG-3′. Specificity of the assay was determined by the amplification of a 5′-flanking region located from −4861 to −4768 of *RNF43* transcriptional start site using the following primers; forward primer, (−4861) 5′- CAAGGCTAGTCTGCCTCCAG-3′, reverse primer, (−4768) 5′- AGCGCTTTCCAAAGGAGGAA-3′. In addition, the amplifications of c-Myc (*MYC*) enhancer was used as a positive control (forward primer, 5′-GCTCAGTCTTTGCCCCTTTGTGG-3′, reverse, 5′- AA-CACCTTCCCGATTCCCAAGTG-3′).

Results

Knockdown of β-catenin Suppresses *RNF43*

To confirm that *RNF43* is regulated by the Wnt/β-catenin pathway, we measured expression levels of *RNF43* with or without β-catenin siRNA in HCT116 and SW480 cells (Figure 1A). HCT116 and SW480 cells exhibited constitutive activation of Wnt/β-catenin pathway through a mutation in *CTNNB1* or *APC*, respectively. Expectedly, quantitative RT-PCR disclosed that *RNF43* transcripts were markedly decreased by the depletion of β-catenin in these cells (Figure 1B). Consistently the protein level of RNF43 was reduced in SW480 cells treated with siCTNNB1 (Figure 1A). RNF43 protein was not detected in HCT116 cells because they harbor a homozygous mutation of *RNF43*. Since *RNF43* transcripts were more markedly decreased by siCTNNB1 in HCT116 than SW480, we used HCT116 cells for the analysis of regulatory region(s).

Promoter Analysis of *RNF43*

To identify the regulatory element(s) of Wnt-signaling in *RNF43*, we constructed three forms of reporter plasmids (RNF43-5′-1, RNF43-5′-2 and RNF43-5′-3) containing the 5′-flanking region and intronic regions of *RNF43*. RNF43-5′-1 contained approximately 1.6 kb (chr17:56494505–56496131, GRCh37), RNF43-5′-2 approximately 2.5 kb (chr17:56493599–56496131, GRCh37) and RNF43-5′-3 approximately 5.1 kb (chr17:56491044–56496131, GRCh37) of *RNF43*, respectively (Fig. 2A). These regions contained two elements, 5′-CTTTGAG-3′and 5′-CTTTGTC-3′, similar to the putative TCF/LEF-binding motifs (CTTTGWW) between −274 and −268, and between −54 and −48 of TSS, respectively. The reporter plasmids were transiently transfected with or without β-catenin siRNA in HCT116 cells, and luciferase activities were measured. As a result, we found that the luciferase activity of the cells transfected with RNF43-5′-1 or RNF43-5′-2 was significantly higher (approximately 3.0 and 2.5-fold, respectively) than that with empty vector, and that the activity of RNF43-5′-3 was similar to the empty vector (Fig. 2B). However, the knockdown of β-catenin did not affect the reporter activity of RNF43-5′-1 or RNF43-5′-2 (Fig. 2C). These data suggest that the 5.1-kb region contains the promoter but it does not include regulatory element(s) associated with Wnt/β-catenin signaling.

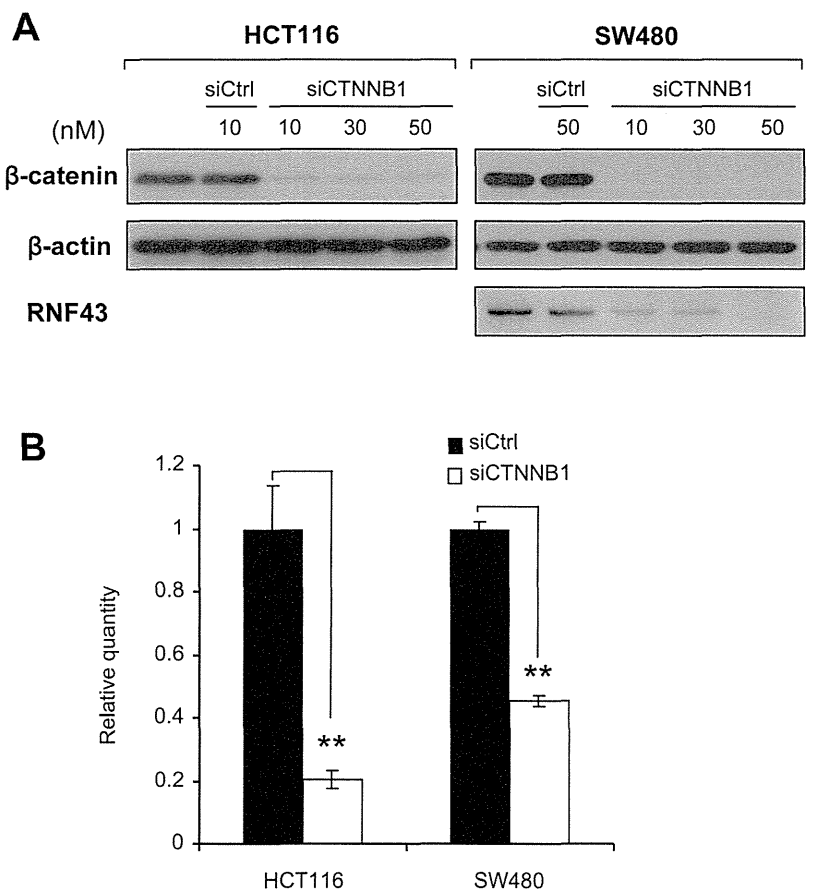

Figure 1. Effect of β-catenin depletion on *RNF43* expression. A) Knock down of β-catenin using *CTNNB1*-specific siRNA (siCTNNB1). HCT116 and SW480 cells were treated with siCTNNB1 or siCtrl, at the concentrations indicated in the figure. Expression levels of β-catenin and RNF43 were detected by western blotting with β-catenin- and RNF43-specific antibodies, respectively. B) Quantitative RT-PCR was carried out in triplicate using RNA from the cells. Cells were treated with 15 nM of *CTNNB1* siRNA (siCTNNB1) or control siRNA (siCtrl) for 48 hours. Relative expression of *RNF43* to the control siRNA is shown (mean ± standard deviation). A significant difference was determined by Student's t-test. **; $P<0.01$.
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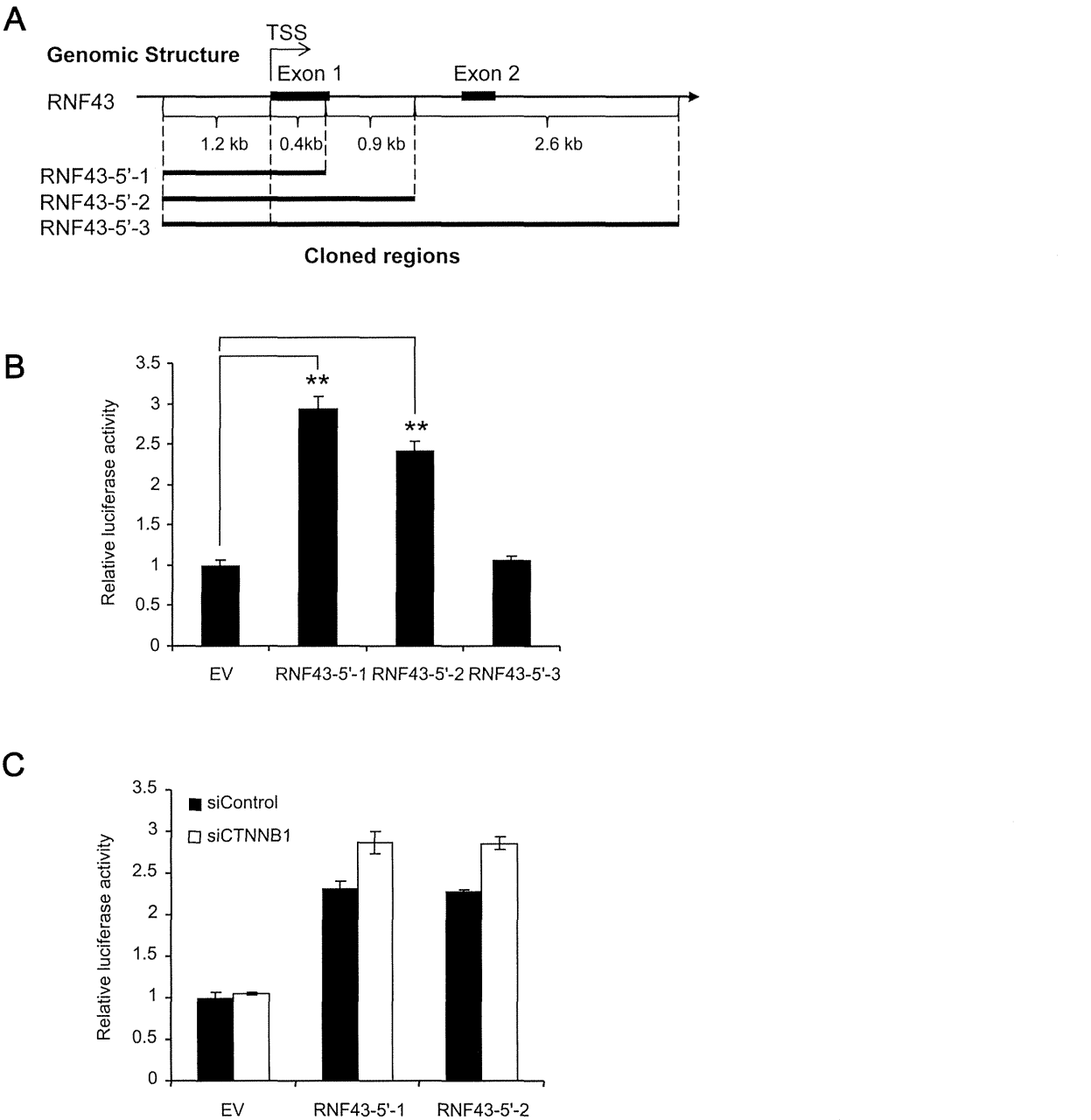

Figure 2. Promoter activity of the TSS-flanking region of *RNF43*. A) A genomic map of the TSS-flanking region of RNF43, and schematic representation of inserted regions in the reporter plasmids (pGL3-basic). TSS: transcription start site. B) Promoter activities of the reporter plasmids (mean ± standard deviation, **; $P<0.01$, Student's t-test). C) Effect of β-catenin depletion on the promoter activities (RNF43-5′-1 and RNF43-5′-2). EV: empty vector.
doi:10.1371/journal.pone.0086582.g002

Identification of WREs in *RNF43* Intron2

We next searched for putative regulatory regions in *RNF43* in public databases. A search in the ChIP-seq data of the ENCODE project (http://www.genome.ucsc.edu; The University of California Santa Cruz Genome Browser Database), identified four TCF4-enriched regions in the *RNF43* gene; one between −517 and +100 of TSS, two in intron2, and one in intron3. The two regions in intron2, but not the one in intron3, overlapped with RNA pol II-enriched regions. It is of note that these three regions were enriched with histone H3K4 mono-methylation. On the other hand, the region between −517 and −100 of TSS was enriched with pol II, histone H3K4 tri-methylation, and histone H3K27 acetylation, but not with histone H3K4 mono-methylation. Therefore we focused on the two regions in intron2 and tested whether they encompass TCF4-mediated transcriptional enhancer(s).

We then carried out a reporter assay using plasmids (RNF43-int2) containing a genomic region of 4.2 kb (chr17:56468435–56472609, GRCh37) encompassing the two TCF4-enriched regions in intron2. As we expected, the plasmids showed approximately 5-fold higher activity than empty promoter vector in HCT116 cells (Figure 3B). Importantly, the activity was significantly decreased by the treatment with β-catenin siRNA (Figure 3C). The β-catenin-dependent activation was also observed in SW480 cells (data not shown). These data strongly suggested that the 4.2 kb region might be involved in the Wnt-dependent transcriptional activation.

An additional search of transcription factor binding sites identified two putative TCF/LEF-binding motifs (CTTTGWW) in the regions; 5′-CTTTGAA-3′ and 5′-CTTTGAT-3′, which were termed putative Wnt-responsive element 1 and 2 (WRE1 and WRE2), respectively (Figure 3A). To clarify which element is crucial for the transactivation of *RNF43*, we prepared two forms of reporter plasmids containing either WRE1 or WRE2 (RNF43-int2-A and RNF43-int2-B, respectively). Although the luciferase

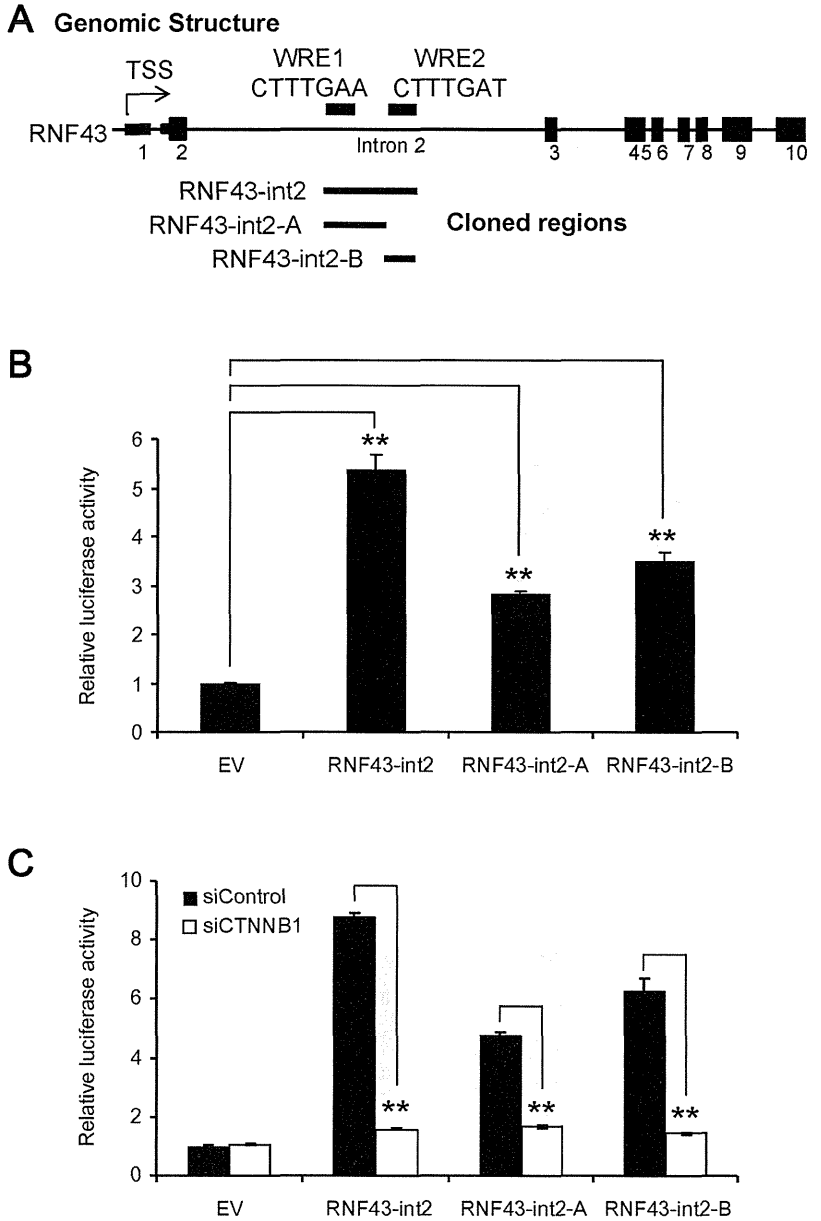

Figure 3. Luciferase assays with different regions in intron2. A) Schematic representation of Tcf4-enriched regions in the ENCODE data, and inserted regions in the promoter vector (pGL3-promoter). WRE: Wnt-responsive element. B) Enhancer activities were measured in triplicate using plasmids (RNF43-int2, RNF43-int2-A, and RNF43-int2-B) containing different regions in intron2 in HCT116 cells (mean ± standard deviation, **; $P<0.01$, Dunnett's test). C) Effect of β-catenin depletion on the enhancer activities (**; $P<0.01$, Student's t-test).
doi:10.1371/journal.pone.0086582.g003

activities of RNF43-int2-A and RNF43-int2-B were decreased at 57% and 65% of RNF43-int2, respectively, their activities were significantly higher than the control plasmids. In addition, co-transfection of β-catenin siRNA significantly suppressed both reporter activities (Figure 3C). We further generated mutant reporter plasmids containing substitution(s) in WRE1 and/or WRE2, from CTTTGWW to CTTTGGC (Figure 4A). Compared to the activity of wild type plasmids (RNF43-int2), reporter plasmids containing either substitution in WRE1 or WRE2 reduced the luciferase activity in HCT116 cells by 34% and 14%, respectively (Figure 4B). Similarly suppressed reporter activities were detected in SW480 cells (Figure 4C). Since combined substitutions in the WRE1 and WRE2 reduced the reporter activity by 36% in HCT116 cells and 71% in SW480 cells, other factor(s) may be involved in the enhanced reporter activity in HCT116 cells. Nevertheless, these data at least suggest that both elements should play a vital role in the β-catenin/TCF-dependent *RNF43* transactivation.

Interaction of WRE1 and WRE2 with TCF4/β-catenin Complex

To confirm whether TCF4 and β-catenin associates with WRE1 and WRE2, we conducted ChIP assays with TCF4-specific or β-catenin-specific antibody in HCT116 cells. Immunoprecipitation and subsequent quantitative PCR analysis revealed that the regions containing WRE1 and WRE2 were enriched by 7.3-fold and 28.1-fold with TCF4-specific antibody, respectively (Figure 5A). Of note, a WRE in the promoter of c-Myc, a direct target of β-catenin/TCF4 complex, was augmented about 13.6-fold in our analysis. Consistently, concordant enrichment of WRE1 and WRE2 was observed with β-catenin-specific antibody (Figure 5B). Similar results were obtained in SW480 cells, although the degree of enrichment was smaller than HCT116 (Figure 5C and 5D). These data indicated that the β-catenin/TCF4 complex interacts with WRE1 and WRE2 in intron2 of *RNF43*.

Discussion

In this study, we have identified regulatory regions of RNF43 transcription and showed that *RNF43* is a direct target gene of Tcf4/β-catenin complex. Our initial challenge to identify regulatory region(s) using reporter assay containing the 5′ flanking region successfully showed that the region is involved in its transcriptional activation, but failed to find region(s) associated with Wnt/β-catenin signaling. Subsequent search of the ENCODE database helped us to explore candidate regions that may interact with Tcf4. Regarding the decrease of reporter activity with RNF43-5′-3 plasmids containing the 5′ flanking region, intron 1, and a part of intron 2, the 3′ region of intron 1 and/or the 5′ region of intron 2 may have a repressive element(s) for the transcription.

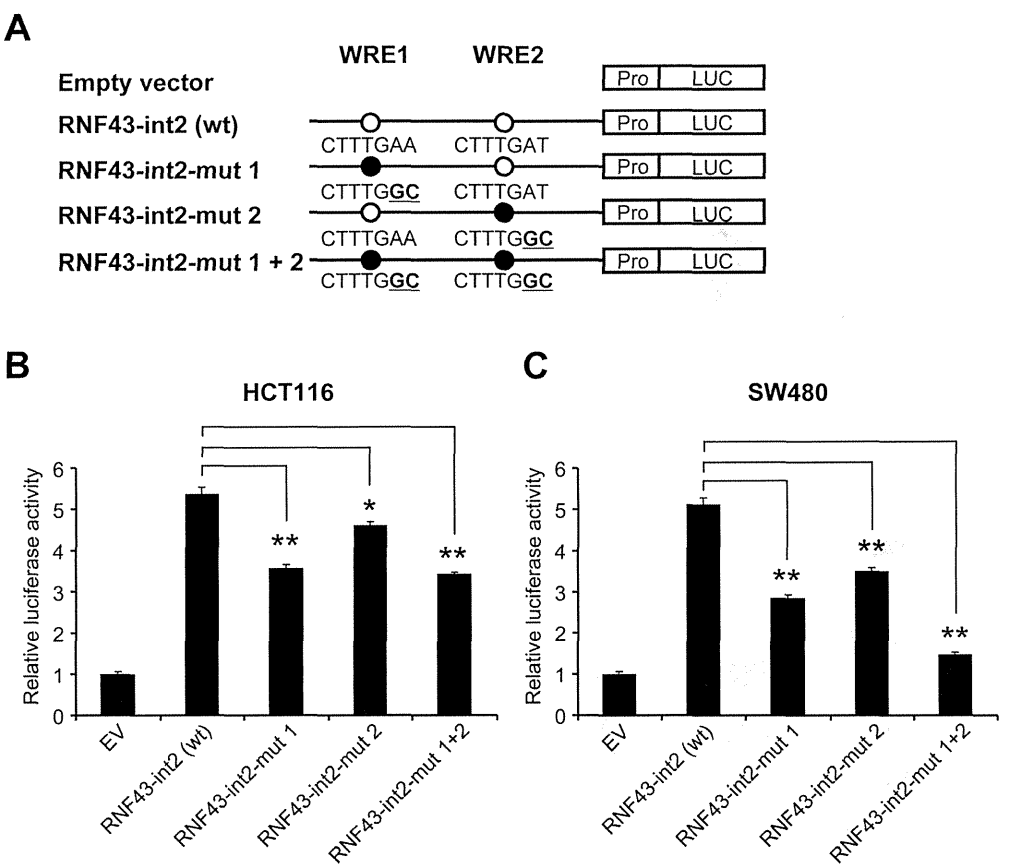

Figure 4. Involvement of WREs in the enhancer activity. A) Schematic representation of reporter plasmids containing substitution(s) in WRE1 and/or WRE2 (RNF43-int2-mut1, RNF43-int2-mut2, and RNF43-int2-mut1+2). B, C) Wild type or mutant reporter plasmids were transfected in HCT116 (B) and SW480 (C) cells, and luciferase activities were measured in triplicate (mean ± standard deviation, *; $P<0.05$, **; $P<0.01$, Dunnett's test).
doi:10.1371/journal.pone.0086582.g004

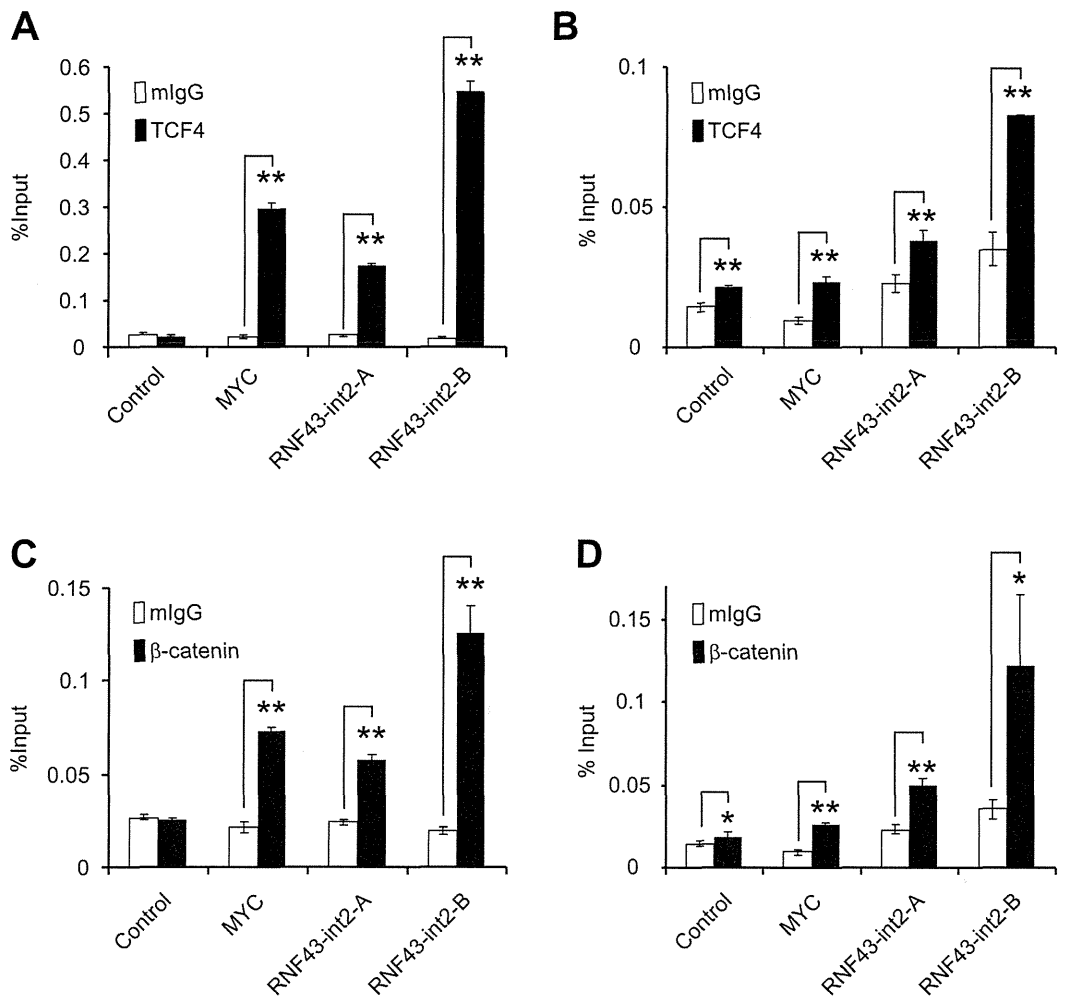

Figure 5. Association of the two WREs with Tcf4 and β-catenin. A, B) Quantification of precipitated regions by a ChIP assay with anti-TCF antibody was performed in HCT116 (A) and SW480 (B) cells using real-time PCR (mean ± standard deviation). Closed boxes indicate ChIP assay with anti-TCF4 antibody, and open boxes with control IgG. C, D) Quantification of precipitated regions by a ChIP assay with anti-β-catenin antibody in HCT116 (C) and SW480 (D) cells. A significant difference was determined by Student's t-test (*; $P<0.05$, **; $P<0.01$).
doi:10.1371/journal.pone.0086582.g005

Genome-wide approaches including ChIP-on-Chip and ChIP-seq analyses have helped to illustrate a global association map of transcription factors, chromatin occupancy, and histone modifications. As for the Tcf4-interacting regions, Hatzis *et al.* found a total of 6,868 enriched regions using tiling array [36]. By means of luciferase-reporter assays, they examined 22 regions of approximately 1000 bp containing at least one of the enriched regions. Consequently, 10 of the 22 increased the activity, and 9 of them were down-regulated by the cotransfection with dominant negative Tcf4, suggesting that the enriched regions do not always play a role in transcriptional activation or associate with Tcf4/β-catenin. Their data included a Tcf4-enriched peak in intron2 of *RNF43* (chr17:53823246, NCBI35/hg17), this peak was close to WRE2 (chr17:53824064–53824070, NCBI35/hg17) suggesting a consistency of transcription factor-binding regions detected by ChIP-on chip in spite of different cell lines they used. They also reported that the Tcf4-binding sites are distributed along the genome, and 18% of them were located in intragenic regions further than 10 kb from the TSS. Consistently, the two WREs of *RNF43* were located at approximately 22 kb downstream of TSS. In addition, their data unveiled that 31% of peaks were located in the 5′-flanking region at a great distance from TSSs. In the case of c-Myc, WREs localized to a region over 400 kb upstream from the gene are involved in chromatin looping in response to the activation with serum mitogens [37]. Therefore additional WREs may play a role for the transcriptional regulation of RNF43 in combination with WRE1 and WRE2.

In addition to Tcf4, genome-wide approaches including serial analysis of chromatin occupancy (SACO) and ChIP-seq have been applied to identify the regions interacting with β-catenin [38,39]. In agreement with our findings, the list of 412 β-catenin-interacting regions detected by SACO included a region containing the Tcf consensus motif in RNF43 (Chr17:53824041 NCBI35/hg17), which corresponded to WRE2 in our data. The same group later carried out a ChIP-seq analysis and identified a total of 2,168 enriched regions with β-catenin in HCT116 cells. In the list of the 2168 regions, three were located in intron2 of RNF43 (chr17:53819951–53820630, chr17:53823671–53824270,

and chr17:53826701–53827401, NCBI36/hg18) [39]. Notably, WRE1 and WRE2 are within two of three enriched regions, suggesting that ChIP-seq is a powerful tool to discover binding regions of transcription factors.

In this study, we incorporated the data of histone modifications and occupancy of RNA pol-II. Consistent with the view that histone H3K4 mono-methylation and an interaction with RNA polymerase II are the hallmarks of transcriptional enhancers, the two WREs in intron2 played a vital role as a transcriptional enhancer in our luciferase assay. Meanwhile, the 5′-flanking region of 2.4 kb was associated with RNA polymerase II and histone H3K4 tri-methylation suggesting that this region served for the constitutive transcriptional activation through the remodeling of heterochromatin complex to euchromatin state. Although the ENCODE data depicted a peak of Tcf4-binding in the 5′-flanking region, enrichment of multiple transcription factors was observed in the same region. Therefore the peak of Tcf4 may be a false positive. Alternatively Tcf4 may interact with that region without the recruitment of β-catenin. Since the data of histone modifications and RNA pol-II occupancy are useful information to predict the chromatin structure of interacting regions and their transcriptional activity, future studies on a global association map of β-catenin and Tcf4 with histone modifications will gain an insight into the dynamic transcriptional regulation played by Tcf4/β-catenin and chromatin modification enzymes such as Brg1, TRRAP, TIP60, CBP/p300, and MLL [10].

Recently, it was reported that RNF43 and ZNRF3, transmembrane E3 ubiquitin ligases, selectively ubiquitinate frizzled receptors and targets them for degradation. RNF43 and ZNRF3, a homologue of RNF43, are highly conserved in vertebrates, and associate in the membrane with frizzled receptors and low density lipoprotein receptor-related proteins, LRP5/6. RNF43 promotes endocytosis of frizzled receptors including FZD1 and FZD3, and suppresses Wnt/β-catenin responses [26]. ZNRF3 also promotes the turnover of frizzled receptors and LRP6. Interestingly inhibition of ZNRF3 enhances Wnt/β-catenin signaling and suppresses Wnt/planar cell polarity signaling [25]. These data suggested that ZNRF3 and RNF43 regulate canonical and non-canonical Wnt pathway. Our data, in line with others, suggest that RNF43 functions as a negative feedback regulator modulated by Tcf4/β-catenin complex. This notion is reminiscent of *AXIN2*, and *DKK1*, which are also downstream targets of the canonical Wnt signaling pathway and negatively regulate the signals in different manners [40,41,42,43,44]. Regarding the tumorigenesis of pancreatic and ovarian cancers, inactivating mutations in *RNF43* are supposed to abrogate Wnt signaling including canonical and non-canonical pathways. However, the effect of negative feedback by the enhanced expression of RNF43 has not been clarified in colorectal or liver cancer. Although the augmented transcriptional activity of Tcf4/β-catenin complex by inactivation mutations in *APC* or *AXIN2*, or activating mutations in *CTNNB1* may not be affected by the suppression of frizzled receptors, complex network of signaling pathways may render undetermined characteristics to colorectal and liver cancer cells, as restoring expression of *SFRP4* and *DKK1* in colorectal cancer cells attenuates Wnt signaling [44,45]. Further investigations on the effect of enhanced or suppressed RNF43 function may shed light on the undetermined networks associated with canonical and non-canonical Wnt pathways, and may contribute to the development of diagnostic, therapeutic, and/or preventive strategies to human diseases.

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Author Contributions

Conceived and designed the experiments: NT YF. Performed the experiments: NT KY. Analyzed the data: NT KY. Contributed reagents/materials/analysis tools: TI TF KY. Wrote the paper: NT YF.

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ORIGINAL ARTICLE

Effect of graft sources on allogeneic hematopoietic stem cell transplantation outcome in adults with chronic myeloid leukemia in the era of tyrosine kinase inhibitors: a Japanese Society of Hematopoietic Cell Transplantation retrospective analysis

Kazuteru Ohashi · Tokiko Nagamura-Inoue · Fumitaka Nagamura · Arinobu Tojo · Kouichi Miyamura · Takehiko Mori · Mineo Kurokawa · Shuichi Taniguchi · Jun Ishikawa · Yasuo Morishima · Yoshiko Atsuta · Hisashi Sakamaki

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Abstract We retrospectively compared transplant outcomes for related bone marrow transplantation (rBMT), related peripheral blood stem cell transplantation (rPBSCT), unrelated bone marrow transplantation (uBMT), and unrelated cord blood transplantation (CBT) in 1,062 patients with chronic myeloid leukemia (CML) aged 20 years or over between January 1, 2000 and December 31, 2009 in Japan. The disease status was as follows: chronic phase 1 (CP1, $n = 531$), CP 2 or later including accelerated phase (CP2-AP, $n = 342$) and blastic crisis (BC, $n = 189$). Graft sources (GS) were rBMT ($n = 205$), uBMT ($n = 507$), rPBSCT ($n = 226$) or CBT ($n = 124$). In multivariate analysis in CP1, lower overall survival (OS) (relative risk [RR]: 6.01, 95 % confidence interval [CI]: 1.20–29.97, $P = 0.029$) and leukemia-free survival (LFS) (RR: 4.26, 95 % CI: 1.24–14.62, $P = 0.021$) were observed in uBMT compared with those in rBMT. For patients in the advanced phase of CML beyond CP1, GS had no significant impact on OS or LFS. Our results support the use of rBMT for adults with CML in CP1, but in contrast to previous reports, the superiority of rPBSCT in advanced stage of CML was not confirmed in our cohorts.

Keywords Chronic myeloid leukemia · Allogeneic hematopoietic stem cell transplantation · Graft sources

On behalf of Choric Myeloid Leukemia Working Group of the Japan Society for Hematopoietic Cell Transplantation.

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K. Ohashi (✉) · H. Sakamaki
Hematology Division, Tokyo Metropolitan Cancer and Infectious Disease Center, Komagome Hospital, Tokyo, Japan
e-mail: k.ohashi@cick.jp

T. Nagamura-Inoue
Department of Cell Processing and Transfusion, Research Hospital, The Institute of Medical Science, The University of Tokyo, Tokyo, Japan

T. Nagamura-Inoue
Japan Cord Blood Bank Network, Tokyo, Japan

F. Nagamura
Division of Clinical Trial Safety Management, Research Hospital, The Institute of Medical Science, The University of Tokyo, Tokyo, Japan

A. Tojo
Department of Hematology/Oncology, Research Hospital, The Institute of Medical Science, The University of Tokyo, Tokyo, Japan

K. Miyamura
Department of Hematology, Japanese Red Cross Nagoya Daiichi Hospital, Nagoya, Japan

T. Mori
Division of Hematology, Department of Medicine, Keio University School of Medicine, Tokyo, Japan

M. Kurokawa
Department of Cell Therapy and Transplantation Medicine, The University of Tokyo, Tokyo, Japan

S. Taniguchi
Department of Hematology, Toranomon Hospital, Tokyo, Japan

J. Ishikawa
Department of Hematology and Chemotherapy, Osaka Medical Center for Cancer and Cardiovascular Diseases, Osaka, Japan

Y. Morishima
Division of Epidemiology/Prevention, Aichi Cancer Center Research Institute, Nagoya, Japan

Introduction

According to the Japan Society for Hematopoietic Cell Transplantation (JSHCT), the number of transplants reported annually for the treatment of CML was 306 in 2000, but drastically dropped to 46 transplants in the year 2009. Unsurprisingly, the drop in transplant activity was observed in Japan after imatinib (IM) became available as an experimental drug in 2000 and subsequently as a frontline treatment for CML in 2001. Thus, the excellent outcomes demonstrated by tyrosine kinase inhibitors (TKIs) argue against the use of allogeneic hematopoietic stem cell transplantation (allo-HSCT) as an upfront therapy for CML in CP1; allo-HSCT is currently recommended for patients with a T315I mutation, or who failed TKIs and progress to advanced phase disease [1–6]. Moreover, the newly launched third generation TKI, ponatinib, having a unique binding mechanism allowing inhibition of BCR-ABL kinases, including those with the T315I mutation may further narrow the range of transplant indication [7, 8]. Therefore, those CML patients who undergo allo-HSCT represent a selection of high-risk patients due to more advanced disease with high rates of accelerated or blast phase. To improve transplant outcomes, comprehensive approaches in transplant strategies including timing, choice of conditioning and GS, maintenance therapy might be needed for those CML patients being selected nowadays for allo-HSCT. The main purpose of this study was to analyze the impact of GS on transplant outcome for patients with CML in the era of TKIs, particularly the role of GS in each disease status. We also clarified the prognostic factors for transplant outcomes in each disease status. We herein report our analysis of 1,062 patients, whose complete registry-based clinical data which were provided by the JSHCT.

Patients and methods

Patients

Data on a total of 1,143 patients of at least 20 years of age who had undergone allogeneic bone marrow, peripheral blood, or cord blood transplantation for CML between January 1, 2000 and December 31, 2009 were initially collected through the Transplant Registry Unified Management Program (TRUMP). Eighty-one patients were excluded from the analysis, because one or two critical data such as alive, relapse, and engraftment status with or without date of onset were missing. Other missing data were dealt as missing data in the study and the analysis numbers in each variable were described, respectively. This included data from the Japan Cord Blood Bank Network (JCBBN), the Japan Marrow Donor Program (JMDP), and JSHCT. These are the 3 largest allo-HSCT registries in Japan, and their roles have been described previously [9]. The study was approved by the data management committees of JSHCT, as well as by the ethical committee of Tokyo Metropolitan Cancer and Infectious Disease Center, Komagome Hospital (Tokyo, Japan), where this study was organized.

Statistical analysis

The outcome endpoints were neutrophil recovery, platelet recovery, acute and chronic GVHD, relapse, transplantation-related mortality (TRM), overall survival (OS), and leukemia-free survival (LFS). The definitions of the statistical models used were in accordance with the statistical guidelines of the European Group for Blood and Marrow Transplantation (EBMT) (http://www.ebmt.org/1WhatisEBMT/whatisebmt2.html). Neutrophil recovery was defined by an absolute neutrophil count (ANC) of at least 0.5×10^9/L for 3 consecutive days, with the first day considered as the recovery day. Platelet recovery was defined by a non-transfused platelet count of at least 20×10^9/L for 3 consecutive days. Deaths occurring before day 90 or day 180 were considered as competing risks for neutrophil or platelet recovery, respectively. The graft failure rate for neutrophils was calculated for patients living without relapse for more than 30 days. Acute and chronic GVHD were diagnosed and graded at each center according to the standard criteria [10–12]. Relapse was defined on the basis of the reappearance of the blast or Philadelphia chromosome (Ph) or *BCR-ABL1* transgene by cytogenetic and/or molecular analysis, including polymerase chain reaction and fluorescence in situ hybridization. TRM was considered a sole cause of non-leukemic deaths occurring after transplantation; OS was defined as the time between transplantation and death due to any cause; LFS was defined as the time interval from allo-HSCT to a first event, either relapse or death, in patients achieving complete remission. HLA antigen disparities were categorised as either GVHD or rejection direction. Low-resolution antigens of HLA-A and HLA-B were identified for all patients by serologic typing or low-resolution molecular typing methods. While, HLA-DRB1 alleles were

Y. Morishima
Japan Marrow Donor Program, Tokyo, Japan

Y. Atsuta
Department of HSCT Data Management/Biostatistics, Nagoya University Graduate School of Medicine, Nagoya, Japan

Y. Atsuta · H. Sakamaki
Japanese Society of Hematopoietic Cell Transplantation, Nagoya, Japan

determined by high-resolution molecular typing using the sequence-based HLA typing method. In rBMT, HLA-DRB1 alleles were counted as identical, if the low-resolution antigens of HLA-A, B, and DR were identical. Data on HLA-DRBI allele were not fully available; there were 2 lacking data in CP1, 4 lacking data on CP2-AP and 2 lacking data in BC. Detail of HLA disparity toward either rejection or GVHD are noted in Table 1 and Supplementary Table 1.

Adjusted probabilities of OS and LFS were analyzed using Cox proportional-hazards regression model. The variables used were patients' age at HSCT, patients' sex, body weight at HSCT, time from diagnosis to HSCT, ABO mismatch, conditioning regimen, imatinib administration, kind of GVHD prophylaxis, and year of HSCT. Variables with more than two categories were dichotomized for the final multivariate analyses. Variables were dichotomized as the followings: patient's age at HSCT younger or older than median; patient's body weight at HSCT lighter or heavier than median; time from diagnosis to HSCT <1 year or >1 year. ABO major mismatch or others; myeloablative conditioning regimen or others; cyclosporine-based GVHD prophylaxis regimen or tacrolimus-based; year of HSCT before or after 2004. The endpoints of neutrophil and platelet recovery, acute GVHD and chronic GVHD, relapse and TRM were analyzed using cumulative incidence curves that estimated incidence according to the Fine and Gray models, in which we first used univariate models that contained each of the variables one at a time. Then all variables with a $P < 0.05$ by the likelihood-ratio test were included in a multivariate model.

Cause-specific hazard ratios were estimated with 95 % confidence intervals (CIs). Statistical analysis was performed with the R Foundation statistical computing package, version 2.12.2 (http://www.r-project.org/).

Table 1 Characteristics of patients with CML in CP1, CP2-AP, and BP

	CP1 ($n = 531$)	CP2-AP ($n = 342$)	BP ($n = 189$)
Graft source rBMT/uBMT/rPBSCT/CBT	138/258/125/10	43/176/59/64	24/73/42/50
Gender	338/193 ($P < 0.001$)	215/127 ($P < 0.001$)	123/66 ($P < 0.001$)
Male/female			
Median age at transplantation (range)	40 (20–67)	43 (21–69)	43 (20–74)
GVHD prophylaxis CyA + MTX/CyA based/FK + MTX/FK based/ others	331/27/144/12/14[a]	148/17/145/19/9[a]	88/22/58/17/2[a]
Pre-transplant IM	133/249[b]	187/108[b]	94/95 ($P = 0.94$)
Yes/no	($P < 0.001$)	($P < 0.001$)	
Duration from diagnosis to transplantation, months median (range)	12.5 (0.8–169.0)	18.2 (1.6–255.3)	15.5 (2.4–322.7)
Duration from diagnosis to transplantation ≤1 year/> 1 year	248/258[c] ($P = 0.65$)	135/195[c]	80/100[c] ($P = 0.14$)
		($P < 0.001$)	
Patient's body weight, kg Median (range)	61 (40–104)	60 (34–104)	58.5 (34–96)
Conditioning regimen Myeloablative/reduced intensity	475/53[d] ($P < 0.001$)	289/53 ($P < 0.001$)	161/28 ($P < 0.001$)
Years at transplantation 2000–2004/2005–2009	447/84 ($P < 0.001$)	211/131 ($P < 0.001$)	116/73 ($P < 0.01$)
ABO mismatch No/yes	189/161[e] ($P = 0.13$)	132/156[e] ($P = 0.16$)	64/91[e] ($P = 0.03$)
HLA disparities (rejection direction)[g] 0–1/> 2	510/19[f] ($P < 0.001$)	281/57[f] ($P < 0.001$)	145/42[f]
			($P < 0.001$)
HLA disparities (GVHD direction)[g] 0–1/> 2	507/22[f] ($P < 0.001$)	285/53[f] ($P < 0.001$)	140/47[f]
			($P < 0.001$)

CP chronic phase, *AP* accelerated phase, *BP* blastic phase, *rBMT* related bone marrow transplantation, *rPBSCT* related peripheral blood stem cell transplantation, *uBMT* unrelated bone marrow transplantation, *CBT* unrelated cord blood transplantation, *GVHD* graft-versus-host disease, *CyA* cyclosporine, *MTX* methotrexate, *FK* tacrolimus, *IM* imatinib mesylate, *HLA* human leukocyte antigen

[a] Data on GVHD prophylaxis were not fully available; there were 3 missing data in CP data, 4 missing data on CP2-AP and 2 missing data in BC

[b] Data on pre-transplant imatinib administration were not fully available; 149 data and 47 data were not retrieved in CP1 and in CP2-AP, respectively

[c] Loss of data on duration from diagnosis to transplantation (≤ 1 year/> 1 year) was noted; 25 data in CP, 12 data in CP2-AP, and 9 data in BP were not retrieved

[d] Three data regarding conditioning regimen in CP were not retrieved

[e] Loss of data on ABO mismatch was noted; 181 data in CP, 54 data in CP2-AP, and 34 data in BP were not retrieved

[f] Data on HLA-DRBI allele were not fully available; there were 2 lacking data in CP, 4 lacking data on CP2-AP and 2 lacking data in BC

[g] More detail of HLA disparity toward either rejection or GVHD is noted in supplementary Table 1

Results

Patient characteristics

Of 1,062 patients (676 men, 386 women; median age, 41 years; range, 20–74), 414 patients (39 %) had a clear history of pre-transplant IM use. Disease status was as follows: CP1 ($n = 531$), CP2-AP ($n = 342$) and BC ($n = 189$). GS were related rBMT ($n = 205$), uBMT ($n = 507$), rPBSCT ($n = 226$) and CBT ($n = 124$). The unrelated PBSCT has not been allowed in Japan until 2012 and, therefore, our data included only unrelated BMT, not PBSCT. In addition, during the study period, there were no related CBTs at all. The other variables, including GVHD prophylaxis, pre-transplant IM, body weight at allo-HSCT, duration from diagnosis to transplant, conditioning intensity, years at transplantation (2000–2004 vs. 2005–2009), ABO mismatch, HLA mismatch in either GVHD or rejection direction, are shown in Table 1.

Overall survival and leukemia-free survival

The median follow-up period was 914 days after transplantation (range 2–3,902) and 1,914 days after diagnosis (range 29–9,120). Three-year OS was 70.6 % (95 % CI, 66.8-74.7 %) for patients in CP1 at the time of transplantation, 58.9 % (95 % CI, 53.7–64.7 %) for those with CP2-AP, and 26.9 % (95 % CI, 20.9–34.6 %) for those in BC. The probability of 3-year LFS for patients in CP1, CP2-AP and BC was 64.6 % (95 % CI, 60.4–68.6 %), 46.1 % (95 % CI, 40.9–51.9 %) and 19.2 % (95 % CI, 14.1–26.1 %), respectively (data not shown).

OS and LFS according to GS in CP1, CP2-AP, and BC are shown in Fig. 1a–c, and d–f, respectively. In view of OS and LFS according to GS, 3-year OS after rBMT, rPBSCT, uBMT, and CBT in CP1 was 84.4, 70.0, 64.4, and 48.0 %, respectively (Fig. 1a). Three-year LFS after rBMT, rPBSCT, uBMT, and CBT in CP1 was 76.3, 64.3, 59.3, and 30 %, respectively (Fig. 2d). Multivariate analysis for OS identified the following factors as adverse prognostic factors for

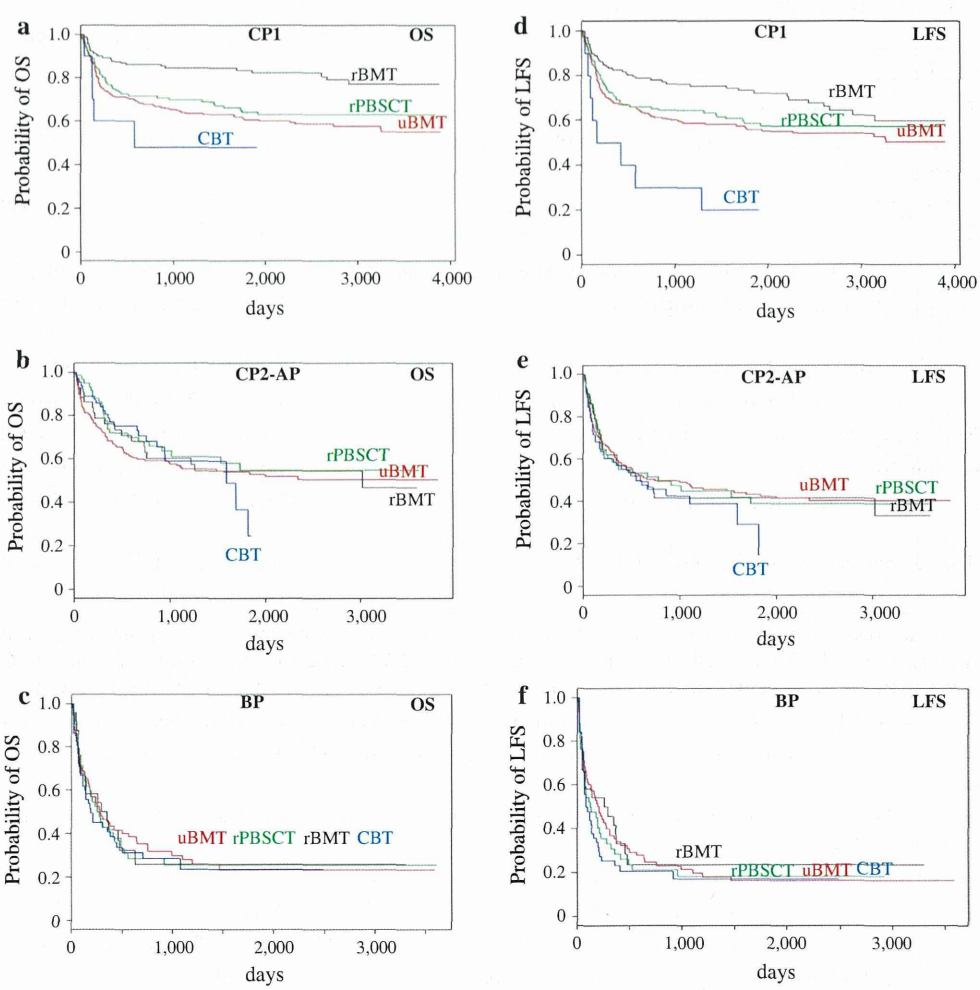

Fig. 1 Kaplan–Meier estimate of overall survival (OS) for patients in CP1 (**a**), CP2-AP (**b**) and BC (**c**); and leukemia-free survival (LFS) for patients in CP1 (**d**), CP2-AP (**e**) and BC (**f**)

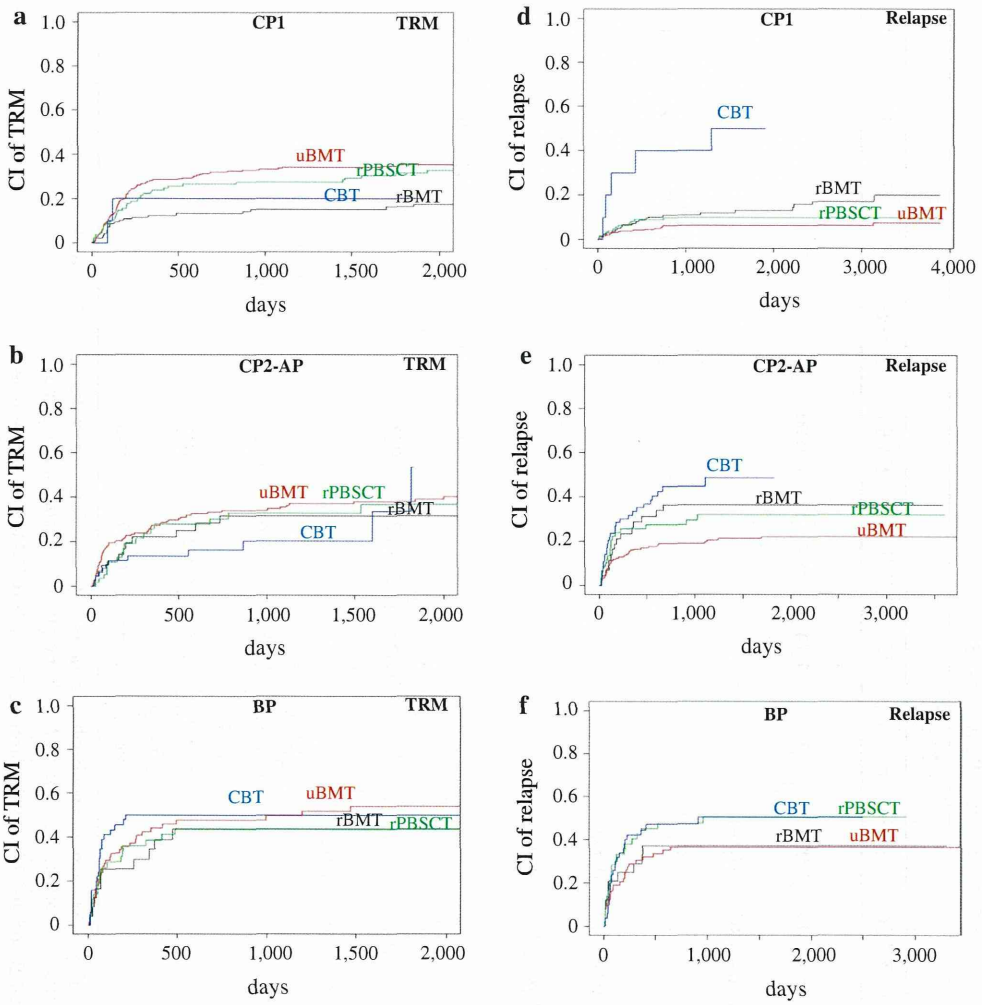

Fig. 2 The cumulative incidence of transplantation-related mortality (TRM) for patients in CP1 (**a**), CP2-AP (**b**) and BC (**c**); and relapse for patients in CP1 (**d**), CP2-AP (**e**) and BC (**f**)

patients in CP1: older age (>median age, 40 years: HR 1.67, 95 % CI, 1.15–2.41, $P = 0.007$), ABO mismatch (HR 1.44, 95 % CI, 1.003–2.06, $P = 0.048$) (Table 2), and uBMT (RR 6.01, 95 % CI, 1.20–29.97, $P = 0.029$) (Table 3). In CP2-AP, older age (> median age, 43 years: HR 1.74, 95 % CI, 1.25–2.43, $P < 0.001$) was the only factor an adverse prognostic factor (Table 2). In BC, pre-transplant IM (HR 0.61, 95 % CI, 0.49–0.89, $P = 0.011$) was the only factor for better OS (Table 2). Concerning LFS, multivariate analysis showed that uBMT (RR 4.26, 95 % CI, 1.24–14.62, $P = 0.021$) and older age (>median age, 40 years: HR 1.43, 95 % CI, 1.02–1.99, $P = 0.038$) were adverse risk factors in CP1 (Table 2, 3). For patients in CP2-AP and BC, no significant factor for OS or LFS was found. Thus, for patients in CP1, GS could have a significant impact on survival outcomes. While, for patients in the advanced phase of CML of beyond CP1, GS could have no significant impact on OS or LFS (Table 3).

TRM and relapse

The 1-year cumulative TRM rate by disease stage was 23.1 % (95 % CI, 19.5–26.7 %) in CP1, 24.2 % (95 % CI, 19.5–28.9 %) in CP2-AP, and 43.2 % (95 % CI, 35.9–50.5 %) in BC. TRM by GS is shown in Fig. 2a–c. The TRM rate appeared low in rBMT compared with uBMT or rPBSCT in CP1 (Fig. 2a). Multivariate analysis showed that uBMT (RR 2.49, 95 % CI 1.02–6.10, $P = 0.046$) and older age (>median age, 40 years: HR 1.69, 95 % CI, 1.19–2.39, $P = 0.003$) were factors associated with a significantly increased risk of TRM in CP1 (Table 2, 3).

The 3-year cumulative relapse rate by disease stage was 9.0 % (95 % CI, 3.9–7.9 %) in CP1, 28.2 % (95 % CI, 23.3–33.1 %) in CP2-AP, and 43.6 % (95 % CI, 36.3–50.9 %) in BC. Relapse rate by GS is demonstrated in Fig. 2d–f. For patients in CP1, the relapse rate after CBT appeared to be higher than that after other GS (Fig. 2d). In multivariate analysis by the effect of GS in CP1, CBT (RR

Table 2 Multivariate analysis of risk factors for the main outcomes after allo-HSCT for CML in CP1, CP2-AP, and BP

Main outcomes	Factors	CP1				CP2-AP				BP			
		Factors	HR	(95 % CI)	*P* value	Factors	HR	(95 % CI)	*P* value	Factors	HR	(95 % CI)	*P* value
OS	Age	≤40	1			≤43	1						
		>40	1.67	1.15–2.41	0.007	>43	1.74	1.25–2.43	< 0.001				
	ABO mismatch	No	1										
		Yes	1.44	1.003–2.06	0.048								
	Pre-transplant IM									No	1		
										Yes	0.61	0.41–0.89	0.011
LFS	Age	≤40	1										
		>40	1.43	1.02–1.99	0.038								
TRM	Age	≤40	1										
		>40	1.69	1.19–2.39	0.003								
Relapse	HLA mismatch (rejection)									0, 1	1		
										≥2	1.7	1.04–2.76	0.033
	HLA mismatch (GVHD)					0, 1	1						
						≥2	3.57	1.55–8.21	0.003				
Acute GVHD (all grades[a])	Pre-transplant IM	No	1										
		Yes	0.75	0.57–0.99	0.04								
	BW					≤60 kg	1						
						>60 kg	1.35	1.01–1.82	0.045				
Acute GVHD	BW					≤60 kg	1						
(≥grade 2)						> 60 kg	1.53	1.05–2.24	0.028				
Chronic GVHD (extensive[b])	BW					≤60 kg	1						
						>60 kg	1.75	1.06–2.73	0.028	0			

OS overall survival, *LFS* leukemia-free survival, *TRM* transplantation-related mortality, *ANC* absolute neutrophil count, *GVHD* graft-versus-host disease, *IM* imatinib, *HLA* human leukocyte antigen, *BW* body weight, *HR* hazard ratio, *CI* confidence interval, *CP* chronic phase, *AP* accelerated phase, *BP* blastic phase, *imatinib* imatinib mesylate

[a] Overall grade of acute GVHD assigned according to the Center for International Blood and Marrow Transplant Research (CIBMTR) severity index

[b] Chronic GVHD was graded as limited or extensive based on the Seattle criteria

Table 3 Impact of graft sources on main outcomes after allo-HSCT for CML in CP1, CP2-AP, and BP

Main outcomes	Graft sources	CP1			CP2-AP			BP		
		RR	(95 % CI)	*p* value	RR	(95 % CI)	*p* value	RR	(95 % CI)	*p* value
OS	rBMT	1.00			1.00			1.00		
	uBMT	6.01	(1.20–29.97)	0.029	1.12	(0.33–3.79)	0.851	>99	(0.00–99.99)	0.999
	rPBSCT	1.76	(0.77–4.04)	0.180	0.84	(0.21–3.43)	0.809	1.13	(0.56–2.30)	0.727
	CBT	1.00	(0.00–99.99)	1.000	NA	NA	NA	NA	NA	NA
LFS	rBMT	1.00			1.00			1.00		
	uBMT	4.26	(1.24–14.62)	0.021	1.61	(0.55–4.74)	0.383	0.00	(0–99.99)	0.999
	rPBSCT	1.72	(0.95–3.11)	0.073	0.42	(0.14–1.31)	0.135	0.67	(0.31–1.44)	0.299
	CBT	1.00	(0.00–99.99)	1.000	NA	NA	NA	NA	NA	NA
TRM	rBMT	1.00			1.00			1.00		
	uBMT	2.49	(1.02–6.10)	0.046	1.36	(0.60–3.09)	0.47	2.71	(0.74–9.96)	0.13
	rPBSCT	1.03	(0.52–2.07)	0.93	0.94	(0.52–1.70)	0.83	1.43	(0.64–3.22)	0.39
	CBT	0.33	(0.04–2.63)	0.29	0.98	(0.60–1.60)	0.94	1.26	(0.82–1.92)	0.29
Relapse	rBMT	1.00			1.00			1.00		
	uBMT	0.33	(0.12–0.95)	0.041	0.66	(0.29–1.55)	0.34	2.23	(0.28–17.61)	0.45
	rPBSCT	1.13	(0.62–2.07)	0.68	1.17	(0.64–2.14)	0.6	1.06	(0.44–2.54)	0.9
	CBT	25.16	(1.76–369.10)	0.018	1.15	(0.74–1.80)	0.53	0.77	(0.39–1.60)	0.49
ANC recovery	rBMT	1.00			1.00			1.00		
	uBMT	0.82	(0.55–1.23)	0.35	0.83	(0.53–1.31)	0.43	0.58	(0.27–1.26)	0.17
	rPBSCT	1.31	(1.02–1.69)	0.036	1.2	(0.90–1.59)	0.21	0.91	(0.33–2.52)	0.86
	CBT	2	(0.67-5.98)	0.22	0.53	(0.42–0.67)	<0.001	0.55	(0.37–0.82)	0.003
Platelet recovery	rBMT	1.00			1.00			1.00		
	uBMT	0.75	(0.46–1.21)	0.24	0.89	(0.51–1.56)	0.68	0.21	(0.07–0.61)	0.0039
	rPBSCT	0.93	(0.69–1.26)	0.65	0.91	(0.61–1.35)	0.63	0.67	(0.28–1.57)	0.35
	CBT	1.07	(0.35–3.28)	0.9	0.78	(0.62–0.99)	0.049	0.44	(0.26–0.74)	0.0018
Acute GVHD (all grades[a])	rBMT	1.00			1.00			1.00		
	uBMT	3.35	(1.50–6.22)	<0.001	1.67	(0.92–3.02)	0.09	1.22	(0.46–3.25)	0.69
	rPBSCT	1.49	(0.94–2.37)	0.091	0.86	(0.51–1.44)	0.56	0.94	(0.32–2.73)	0.91
	CBT	1.67	(0.68–4.11)	0.26	0.76	(0.58–1.01)	0.054	1.05	(0.56–1.96)	0.87
Acute GVHD (≥grade 2)	rBMT	1.00			1.00			1.00		
	uBMT	4.28	(1.92–9.53)	<0.001	2.14	(0.93–4.94)	0.075	1.34	(0.39–4.61)	0.65
	rPBSCT	1.5	(0.82–2.72)	0.19	1.53	(0.82–2.86)	0.18	2.23	(0.36–1.39)	0.39
	CBT	1.00	(0.00–99.99)	1.000	0.84	(0.58–1.22)	0.36	1.45	(0.55–3.81)	0.45
Chronic GVHD	rBMT	1.00			1.00			1.00		
	uBMT	0.95	(0.53–1.70)	0.86	1.1	(0.45–2.68)	0.84	0.27	(0.06–1.33)	0.11
	rPBSCT	1.37	(0.97–1.92)	0.075	1.24	(0.70–2.19)	0.47	0.84	(0.22–3.20)	0.8
	CBT	8.52	(0.64–11.43)	0.11	0.8	(0.52–1.25)	0.33	0.73	(0.32–1.66)	0.46
Chronic GVHD (extensive[b])	rBMT	1.00			1.00			1.00		
	uBMT	1	(0.49–2.04)	1	0.84	(0.33–2.15)	0.72	0.69	(0.14–3.46)	0.65
	rPBSCT	1.31	(0.87–1.96)	0.19	1.19	(0.60–2.34	0.62	1.08	(0.27–4.24)	0.92
	CBT	6.61	(0.22–200.8)	0.28	0.63	(0.36–1.09)	0.097	0.77	(0.31–1.88)	0.56

OS overall survival, *LFS* leukemia-free survival, *TRM* transplantation-related mortality, *ANC* absolute neutrophil count, *GVHD* graft-versus-host disease, *RR* relative risk, *CI* confidence interval, *CP* chronic phase, *AP* accelerated phase, *BP* blastic phase, *rBMT* related bone marrow transplantation, *rPBSCT* related peripheral blood stem cell transplantation, *uBMT* unrelated bone marrow transplantation, *CBT* unrelated cord blood transplantation, *NA* not available

[a] Overall grade of acute GVHD assigned according to the Center for International Blood and Marrow Transplant Research (CIBMTR) severity index

[b] Chronic GVHD was graded as limited or extensive based on the Seattle criteria

25.16, 95 % CI 1.76–369.10, $P = 0.018$) showed higher relapse, while uBMT (RR 0.33, 95 % CI 0.12–0.95, $P = 0.041$) was lower relapse compared with those in rBMT (Table 3).

Engraftment

The cumulative neutrophil recovery rate on day 90 was 97.5 % (95 % CI, 96.1–98.9 %) in CP1, 93.2 % (95 % CI, 90.5–95.9 %) in CP2-AP, and 82.3 % (95 % CI, 76.8–87.8 %) in BC. On day 180, the cumulative platelet recovery rate, as indicated by more than 2×10^{10}/L of platelets in blood, was 91.9 % (95 % CI, 89.5–94.3 %) in CP1, 85.1 % (95 % CI, 81.2–89.0 %) in CP2-AP, and 67.2 % (95 % CI, 60.3–74.1 %) in BC. Note that the neutrophil recovery and platelet recovery rates were lower after CBT, especially in patients in the advanced phase; i.e., neutrophil recovery in CBT: 90 % in CP1, 79.4 % in CP2-AP, and 64.0 % in BC; platelet recovery after CBT: 90.0 % in CP1, 72.5 % in CP2-AP, and 52.0 % in BC (Fig. 3a–f). Multivariate analysis showed that rPBSCT (RR 1.31, 95 % CI 1.02–1.69, $P = 0.0396$ was a significant factor for early neutrophil recovery in CP1. While, CBT (RR 0.53, 95 % CI 0.42–0.67, $P < 0.001$) was a significant factor for delayed neutrophil recovery in CP2-AP (Table 3). The factor statistically associated with delayed platelet recovery was CBT in CP2-AP (RR 0.78, 95 % CI 0.62–0.99, $P = 0.0049$) and in BC (RR 0.44, 95 % CI 0.26–0.74, $P = 0.0018$). Unrelated BMT (RR 0.21, 95 % CI 0.07–0.61, $P = 0.0039$) was also a significant factor for delayed platelet recovery in BC (Table 3).

Acute and chronic GVHD

The cumulative incidence of acute GVHD at all grades before day 100 was 62.8 % (95 % CI, 58.6–67.0 %) in CP1, 63.5 % (95 % CI, 58.2–58.8 %) in CP2-AP, and 68.6 % (95 % CI, 61.3–74.9 %) in BC. Patients who underwent uBMT showed a higher incidence of acute GVHD (all grades) in CP1 and CP2-AP (Fig. 4a, b). This association was confirmed by multivariate analysis; uBMT (RR 3.35, 95 % CI 1.50–6.22, $P < 0.001$) was a significant factor in CP1 (Table 3). Pre-transplant IM (HR 0.75, 95 % CI 0.57–0.99, $P = 0.04$) was a significant risk factor for acute GVHD (all grades) in CP1 (Table 2). Focusing exclusively on grade II or higher acute GVHD, uBMT (RR 4.28, 95 % CI 1.92–9.53, $P < 0.001$) (Table 3) was a significant risk factor in CP1 (Table 2). For patients in CP2-AP, body weight (>60 kg) was a factor significantly associated with increased risk of aGVHD (all grade; RR 1.35, 95 % CI, 1.01–1.82, $P = 0.045$, grade II or higher grade; RR 1.53, 95 % CI, 1.05–2.24, $P = 0.028$) (Table 2).

The cumulative incidence of chronic GVHD among evaluable patients who survived at least 100 days after allo-HSCT was 49.4 % (95 % CI, 44.7–54.1 %) in CP1, 42.2 % (95 % CI, 36.4–48.0 %) in CP2-AP, and 37.8 % (95 %CI, 30.0–45.6 %) in BC. For patients in CP1, rPBSCT showed a higher incidence of chronic GVHD (71.4 %), which was compared to other GS (Fig. 4d); however, this significant association was not confirmed in multivariate analysis (rPBSCT: RR 1.37 95 % CI 0.97–1.92, $P = 0.075$). For patients in CP2-AP and BC, chronic GVHD after CBT occurred at rates of 23.1 and 23.8 %, respectively, which were apparently lower than that of other GS (Fig. 4e, f), but these statistical associations were not also confirmed by multivariate analysis in CP2-AP or BC (Table 3). Concerning extensive chronic GVHD, multivariate analysis showed the significant association between body weight (>60 kg; RR 1.75, 95 % CI, 1.06–2.73, $P = 0.028$) and chronic GVHD in CP2-AP (Table 2).

Discussion

Our study reviewed 1,062 Japanese adult patients who underwent allo-HSCT during the past decade (2000–2009); thus, our cohort reflects the current use and results of allo-HSCT for CML in Japan. Moreover, the TRUMP database offers the advantage of a large number of patients with extensive data, which permits multivariate analysis. The 3-year OS was 70.6 % for patients in CP1, and the probability of 3-year LFS for patients in CP1 was 64.6 %. These survival data for patients in CP1 were comparable to those reported by others [12]. Based on the report from the EBMT, which included 13,416 CML patients and was apparently the largest CML transplant database including the 3 times cohorts (i.e., 1980–1990, 1991–1999, 2000–2003), the probability of OS at 2 years for patients transplanted in CP1 from an HLA-identical sibling was 74 %, with a cumulative incidence of TRM at 2 years of 22 % and of relapse of 18 % among the most recent cohort transplanted between 2000 and 2003 ($n = 3{,}018$) [13]. The Center for International Blood and Marrow Transplant Research (CIBMTR) recently reported the transplant outcomes of 449 patients with advanced phase CML; the disease-free survival rates remained as low as 35–40 % for CP2, 26–7 % for AP, and 8–11 % for BC [14]. Our series including 432 cases of CP2-AP and 189 cases of BC showed similar survival rates, as the probabilities of 3-year LFS in CP2-AP and BC were 46.1 and 19.2 %, respectively.

Our primary object in this study was to assess the clinical impact of GS according to each disease status. Our study results revealed that the patients in CP1 who were

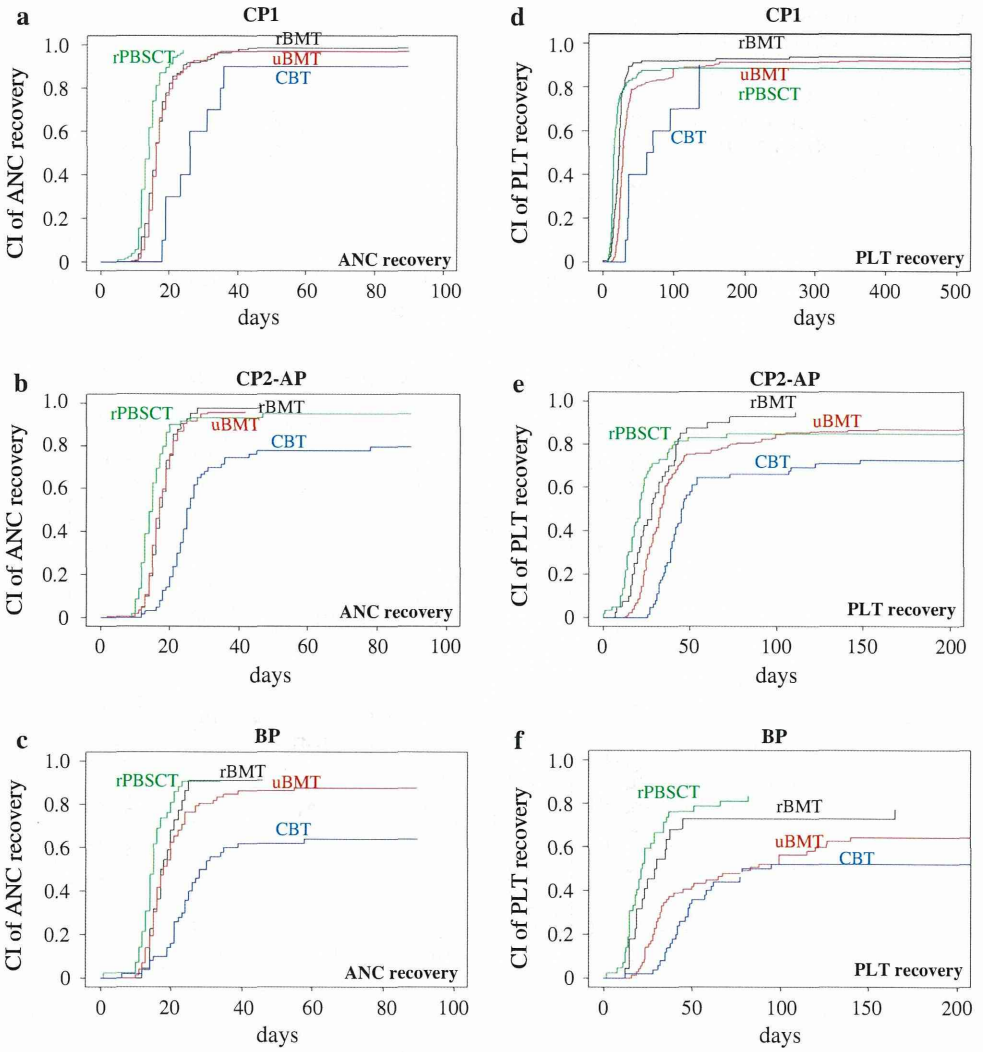

Fig. 3 The cumulative incidence of absolute neutrophil count (ANC) recovery for patients in CP1 (**a**), CP2-AP (**b**) and BC (**c**); and platelet (PLT) recovery for patients in CP1 (**d**), CP2-AP (**e**) and BC (**f**)

treated by rBMT showed a better 3-year OS (84.4 %) with a lower 1-year cumulative incidence of TRM, but the 3-year LFS and relapse rates were similar between patients receiving rBMT and patients receiving rPBSCT. These data were essentially in line with previous reports in which the CIBMTR reported the data of CML patients undergoing rPBSCT or rBMT in CP1; the 1-year LFS and relapse rates were similar for patients receiving rBMT or rPBSCT [14]. We also assessed the clinical impact of GS in CP2-AP; our results showed that there were no significant differences in OS or LFS between GS, despite lower probabilities of relapse after uBMT and lower probabilities of TRM after CBT. These results differ from the IBMTR reports in that for patients in CP2 or AP, rPBSCT was associated with a lower incidence of treatment failure and a higher probability of LFS at 1 year [15]. Regarding GVHD, a recent prospective randomized trial showed a trend toward a higher incidence of chronic GVHD after rPBSCT (59 % after rPBSCT vs. 40 % after rBMT, $P = 0.11$) for patients in CP1 [16]. Our results may confirm this report; although multivariate analysis in our study showed that rPBSCT (RR 1.37 95 % CI 0.97–1.92, $P = 0.075$) was not a significant risk factor for developing chronic GVHD (Table 3), rPBSCT showed a higher incidence of chronic GVHD (71.4 %), which was compared to other GS in CP1 (Fig. 4d).

Several investigators have addressed the clinical impact of pre-transplant IM on post-transplant outcomes for CML [14, 17–20]. The CIBMTR data demonstrated that pre-transplant IM was associated with better survival, but revealed no statistically significant differences in TRM, relapse, and LFS for patients in CP1 [17]. Among patients transplanted in the more advanced phases beyond CP1, pre-transplant IM was not associated with TRM, relapse, LFS, OS, or acute GVHD [17]. In contrast to these studies, our analysis showed that pre-transplant IM was significantly associated with better OS for patients in BC. In addition, multivariate analysis found pre-transplant IM was a