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Cancer Immunology Research

Priority Brief

Quantitative Effect of Natural Killer–Cell Licensing on Hepatocellular Carcinoma Recurrence after Curative Hepatectomy

Naoki Tanimine[1,2], Yuka Tanaka[1,2], Tsuyoshi Kobayashi[1,2], Hirotaka Tashiro[1,2], Daiki Miki[2,3], Michio Imamura[2,4], Hiroshi Aikata[2,4], Junko Tanaka[2,5], Kazuaki Chayama[2,3,4], and Hideki Ohdan[1,2]

Abstract

Natural killer (NK) cells have a potential role in immune surveillance of hepatocellular carcinoma (HCC). Self-recognition of human leukocyte antigens (HLA) through killer immunoglobulin-like receptors (KIR) confers competence to NK cells—a process termed "licensing." We investigated the effect of NK-cell licensing on the susceptibility of patients to HCC recurrence. A total of 170 Japanese patients with HCC who underwent primary curative hepatectomy between 1996 and 2010 were enrolled in this study. The median follow-up period was 5.4 years. We analyzed their KIR-HLA genotypes with sequence-specific polymorphism-based typing and estimated their susceptibility to HCC recurrence by performing propensity score–matching analyses. The presence of KIR2DL1-C2, KIR2DL2-C1, KIR3DL1-BW4, or KIR3DL2-A3/11, functional compound genotypes that intrinsically license NK cells, did not markedly affect HCC recurrence. However, the multiplicity of those compound KIR-HLA genotypes was significantly associated with the HCC recurrence rate, i.e., the cumulative risk of recurrence in patients with at least three compound genotypes was significantly lower than that in patients with one or two compound genotypes, suggesting that the effect of NK-cell licensing on HCC recurrence is quantitative. Patients at high risk of HCC recurrence after curative hepatectomy could be identified by KIR-HLA genotyping. *Cancer Immunol Res; 2(12); 1142–7. ©2014 AACR.*

Introduction

Hepatocellular carcinoma (HCC) frequently recurs despite curative resection (1). Because augmented cytolytic activities of natural killer (NK) cells in the liver are thought to be critical for HCC immune surveillance (2, 3), functional NK-cell competence potentially affects HCC recurrence and prognosis.

NK-cell activation is dependent upon inhibitory-activating receptor equilibrium, among which killer immunoglobulin-like receptors (KIR) are the most polymorphic. KIRs contribute to receptor–ligand interactions that determine NK-cell responses by recognizing specific human leukocyte antigen (HLA) class I allotype ligands (4). Self-specific inhibitory KIR and cognate HLA ligand interactions are fundamental to "licensing" (5), a process in which NK cells expressing inhibitory KIRs for self-HLA have a higher resting response capacity (6). Ligand specificities for five inhibitory KIRs have been defined: KIR2DL1 for the HLA-C Lys80 (C2) group of alleles, KIR2DL2 and KIR2DL3 for the HLA-C Asn80 (C1) group, KIR3DL1 for the Bw4 group of HLA-B (and some A) alleles, and KIR3DL2 for the HLA-A3/11 alleles (7). The genes for KIR and their cognate HLA ligands display extensive polymorphism and generate diverse immune responses to neoplastic cells. Here, we show that the multiplicity of functional compound KIR-HLA genotypes influences posthepatectomy recurrence.

[1]Gastroenterological and Transplant Surgery, Institute of Biomedical and Health Sciences, Hiroshima University, Hiroshima, Japan. [2]Liver Research Project Center, Hiroshima University, Hiroshima, Japan. [3]Laboratory for liver Disease, RIKEN Center for Genomic Medicine, Hiroshima, Japan. [4]Medicine and Molecular Science, Institute of Biomedical and Health Sciences, Hiroshima University, Hiroshima, Japan. [5]Epidemiology, Infectious Disease Control and Prevention, Institute of Biomedical & Health Sciences, Hiroshima University, Hiroshima, Japan.

Corresponding Author: Hideki Ohdan, Department of Gastroenterological and Transplant Surgery, Institute of Biomedical and Health Sciences, Hiroshima University, 1-2-3 Kasumi, Minami-ku, Hiroshima 734-8551, Japan. Phone: 81-82-257-5220; Fax: 81-82-257-5224; E-mail: hohdan@hiroshima-u.ac.jp

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Patients and Methods

Patients and outcomes

A total of 170 Japanese patients with HCC who underwent primary hepatectomy at Hiroshima University between 1996 and 2010 were enrolled in this study based on the following inclusion criteria: Presence of histologically confirmed HCC by an expert pathologist; preserved preoperative liver function, i.e., Child-Pugh grade A; no residual tumor after surgery; no evidence of comorbid malignant tumor; and written informed consent. None of the patients received adjuvant HCC therapy. This study was approved by the Hiroshima University Research Ethics Committee, and informed consent was obtained from all patients in accordance with the Declaration of Helsinki.

Clinicopathologic and follow-up data were collected for 5 years after primary hepatectomy. After hepatectomy, patients were followed up by using ultrasound sonography, contrast-enhanced computed tomography, or magnetic resonance,

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combined with evaluation of serum α-fetoprotein and Des-γ carboxyprothrombin levels at 3-month intervals for up to 3 years. Thereafter, follow-up was performed at 6-month intervals for up to 5 years. HCC recurrence was defined as the appearance of a new focal liver lesion with typical characteristics: lymph node enlargement in the liver hilum or suspected extrahepatic lesions. The diagnosis was histologically confirmed if necessary. Cumulative risk of recurrence was defined as the time from the surgery date to the first tumor recurrence date. Overall survival (OS) was defined as the time from the surgery date to the date of death from any cause.

KIR and HLA genotyping

Genomic DNA was extracted from peripheral blood mononuclear cells derived from patients by using a QIAamp DNA Blood Mini Kit (Qiagen). KIR allele genotyping for KIR2DL1/2DL2/2DL3/3DL1/3DL2 was performed by sequencing KIR transcripts and detected by the reverse sequence-specific polymorphism-polymerase chain reaction (SSP-PCR)–Luminex typing method using a KIR genotyping SSO kit (One Lambda). HLA-A, HLA-B, and HLA-C alleles were identified by SSP-PCR using a WAKFLow HLA typing kit (Wakunaga). The presence of the HLA ligand for KIR was determined according to HLA genotypes, as previously described (8, 9).

Statistical analysis

A comparison of categorical and continuous variables was performed using the χ^2 test and the Wilcoxon test, respectively. To adjust for differences in baseline characteristics, one-to-two or two-to-one propensity score models were constructed on the basis of each patient's estimated propensity score. Variables used included age, sex, etiology [hepatitis B virus/hepatitis C virus (HCV)/others], number of tumors (1, 2, 3, or ≥4), maximum tumor diameter (≤20 mm, >20 mm and ≤50 mm, or >50 mm), histologic type (G1 or G2/G3), vascular invasion (negative/positive), satellite lesions (negative/positive), and surgical margin (<5 mm/≥5 mm). Propensity score matching was performed using IBM SPSS Statistics 18 (SPSS Inc.) and R statistical software version R2.10.0 (R Foundation for Statistical Computing; ref. 10). One-to-two or two-to-one nearest-neighbor matching were performed using a noncaliper.

We considered the 5-year cumulative risk of recurrence as a primary outcome. Cumulative risk of recurrence and OS were estimated and compared using Kaplan–Meier and log-rank statistics. The Cox proportional hazards model was used to calculate the hazard ratio (HR) and 95% confidence intervals (CI). Statistical analyses, except propensity score matching, were performed using JMP10 for Windows (SAS Institute). P values of <0.05 were considered statistically significant.

Results

In this study, 170 patients with HCC who underwent curative hepatectomy were enrolled. Because preoperative liver dysfunction is a risk factor for postoperative HCC recurrence (11, 12), patients with Child-Pugh grade A were included

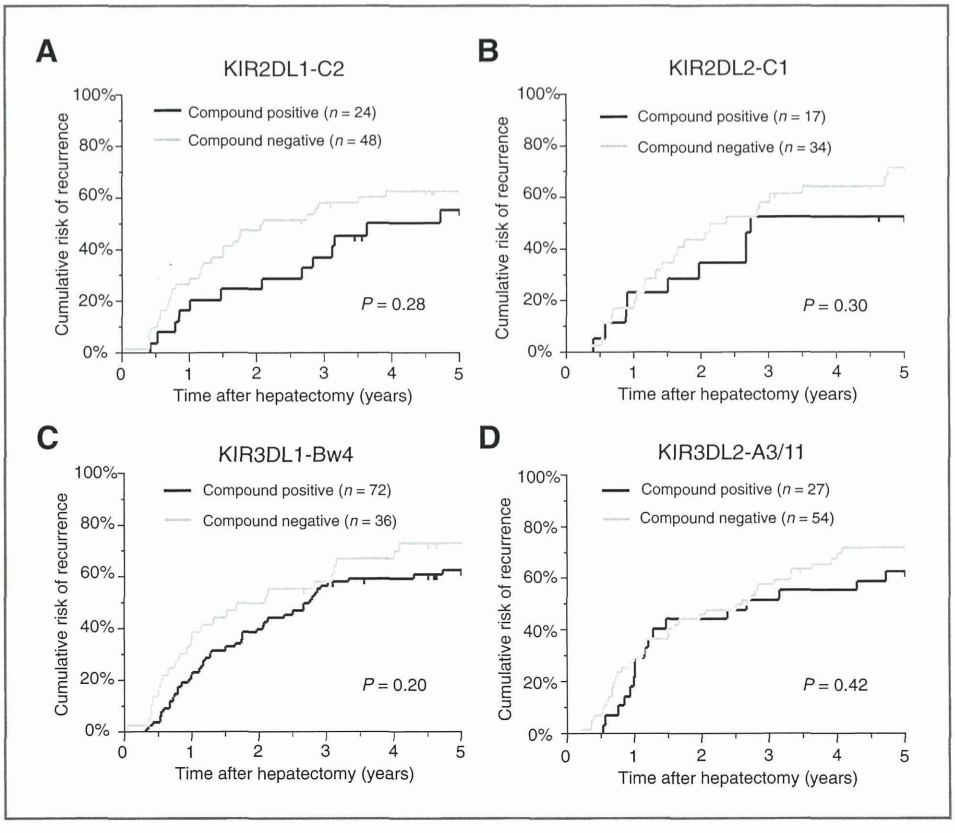

Figure 1. The presence of a single functional compound of KIR-HLA genotype did not affect HCC recurrence. The effect of each compound genotype (KIR2DL1-C2, KIR2DL2-C1, KIR3DL1-BW4, KIR3DL2-A3/11) on HCC recurrence was analyzed by propensity score–matched studies using nine variables (age, sex, etiology, tumor number, maximum diameter, histologic differentiation, vascular invasion, satellite lesion, and surgical margin). The 5-year cumulative risk of recurrence was compared between one-to-two or two-to-one patient pairs with or without each compound KIR-HLA genotype. A, KIR2DL1-C2; B, KIR2DL2-C1; C, KIR3DL1-Bw4; and D, KIR3DL2-A3/11.

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(Supplementary Table S1). The median follow-up period and median OS were 5.4 and 9.1 years, respectively.

The functional compound KIR-HLA genotypes KIR2DL1-C2, KIR2DL2-C1, KIR2DL3-C1, KIR3DL1-Bw4, and KIR3DL2-HLA A3/11, which intrinsically license NK cells, were found in 14.1%, 10.0%, 98.2%, 80.0%, and 15.9% of the cohort, respectively (Supplementary Table S2). The relatively low KIR and HLA genotype heterogeneity agreed with that previously reported (13).

We analyzed the effect of each compound genotype (KIR2DL1-C2, KIR2DL2-C1, KIR3DL1-BW4, and KIR3DL2-A3/11) on HCC recurrence. The KIR2DL3-C1 was present in nearly all patients. Propensity score–matched studies using nine variables (age, sex, etiology, tumor number, maximum diameter, histologic differentiation, vascular invasion, satellite lesion, and surgical margin), in which one-to-two or two-to-one patient pairs with or without each compound KIR-HLA genotype were created, minimized baseline characteristics bias (Supplementary Table S3). Those propensity score–matched analyses revealed that none of the compound KIR-HLA genotypes had a statistically significant effect on post-operative HCC recurrence and OS, although the recurrence rate in the group with each functional compound KIR-HLA genotype was lower than that in the group without that particular genotype (Fig. 1 and Supplementary Fig. S1).

Because NK cells expressing greater numbers of self-reactive inhibitory receptors have increased responsive potential (14), we questioned whether functional compound KIR-HLA genotype multiplicity influenced HCC recurrence. All patients had between one and four of the five functional compound KIR-HLA genotypes. Accordingly, the patients were divided into four groups for risk recurrence comparison. Compound KIR-HLA genotype multiplicity tended to be associated with the HCC recurrence rate and OS (Fig. 2A and B). In the propensity score–matched study using the same nine variables, in which one to two pairs of patients with at least three compound genotypes (the highly licensed NK group; $n = 46$) and patients with one or two compound genotypes (the poorly licensed NK group; $n = 92$) were created (Supplementary Table S4), it revealed that the cumulative recurrence risk in the highly licensed NK group was significantly lower than that in the poorly licensed NK group ($P = 0.018$; adjusted HR, 0.57; Fig. 2C). Likely because treatments against recurring HCC were persistently maintained, no statistical difference was found in OS between the two groups (Fig. 2D).

Subgroup analysis based on tumor–node–metastasis (TNM) classification (7th edition of Union for International Cancer Control) demonstrated that the difference in the cumulative risk of recurrence between the highly and poorly licensed NK groups was consistently recognized in stages I and II (Supplementary Fig. S2A–S2C). No difference was observed between the two groups in stage IIIA (Supplementary Fig. S2D), indicating that the surveillance function of NK cells is most critical in the early stages of HCC. Considering the possible effect of HCV infection on NK-cell activity, additional subgroup analyses were performed among patients with or without HCV. The lower cumulative risk of recurrence in the highly licensed NK group was statistically significant in the non–HCV-related cohort, but not in the HCV-related cohort ($P = 0.044$ and 0.17,

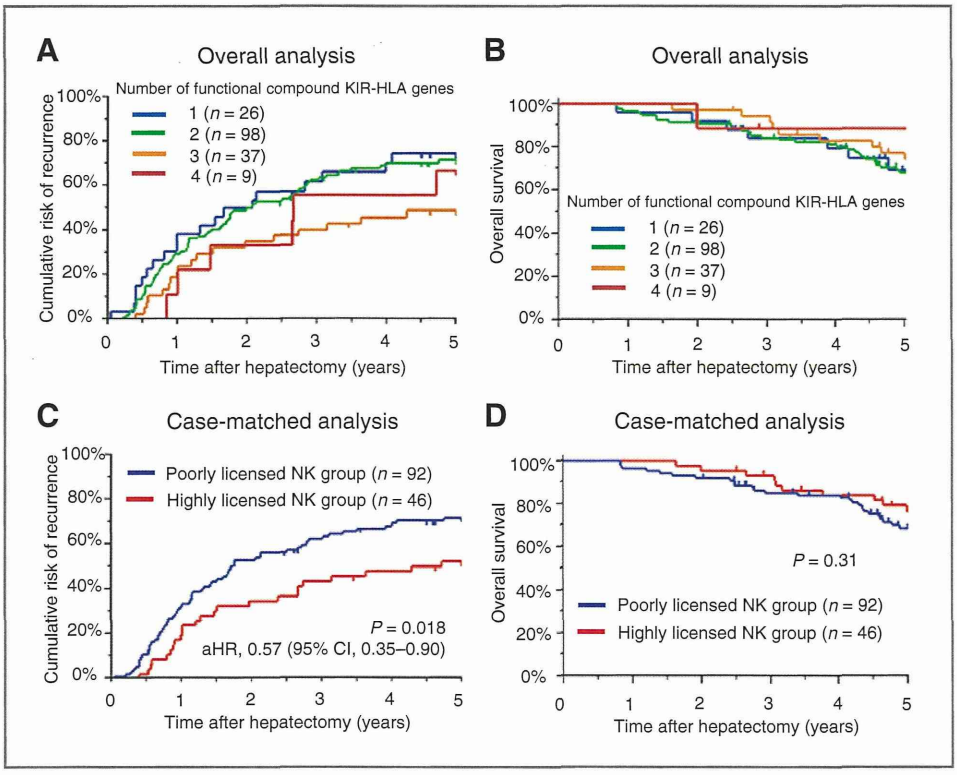

Figure 2. The multiplicity of compound KIR-HLA genotypes stratified the recurrence risk of HCC. Kaplan–Meier analyses of the 5-year cumulative risk of recurrence (A) and OS (B) for 170 patients were performed according to the number of functional compound KIR-HLA genotypes. Propensity score–matched analyses of the 5-year cumulative risk of recurrence (C) and OS (D) were also performed for patients with at least three compound genotypes (highly licensed NK group) and patients with one or two compound genotypes (poorly licensed NK group). The cumulative risk of recurrence in the highly licensed NK group was significantly lower than in the poorly licensed NK group. aHR, adjusted HR.

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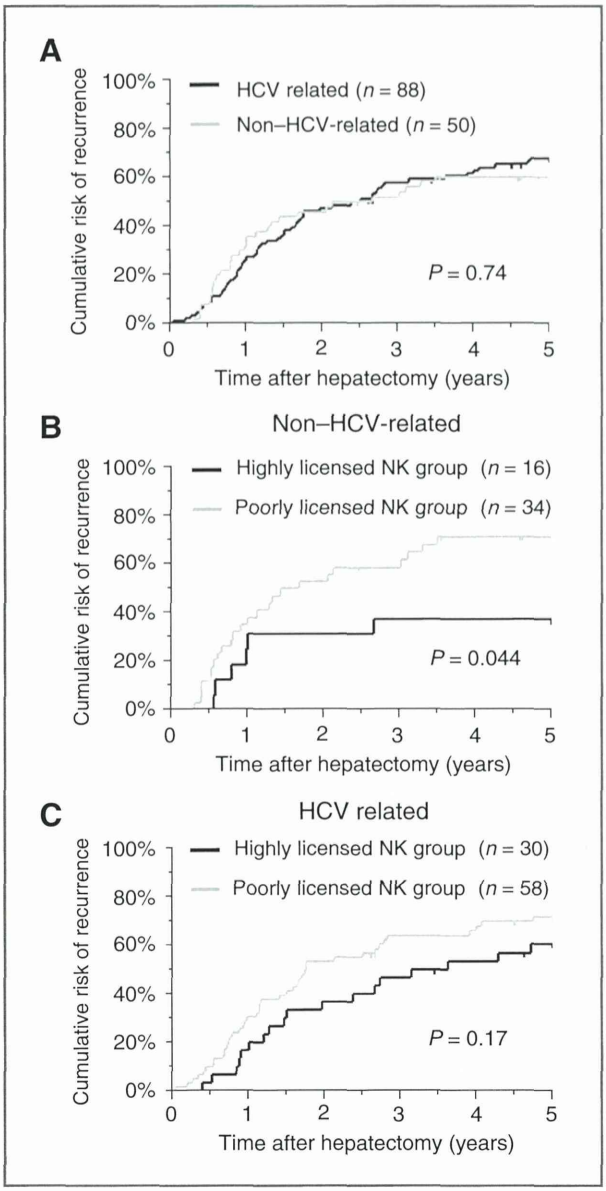

Figure 3. The lower cumulative risk of recurrence in the highly licensed NK group was significant among the non–HCV-related cohort. A, according to the presence or absence of HCV infection, Kaplan–Meier analyses of the 5-year cumulative risk of recurrence were performed for 138 matched patients belonging to the highly and poorly licensed NK groups. Further subgroup analyses were performed in non–HCV-related patients (B) and HCV-related patients (C).

respectively; Fig. 3). Consistently, on the log-rank and the Cox proportional hazards model analyses, the number of KIR-HLA genotype ($\leq$2) was defined as a significant risk factor for HCC recurrence in an unadjusted overall cohort, but was not so in the HCV-related cohort (Table 1).

Discussion

Postoperative recurrent HCC can be monocentric, leading to intrahepatic metastasis, or multicentric as a *de novo* carcinogenesis. To study the role of NK cells in intrahepatic metastasis, we previously investigated the effect of decreasing NK-cell functions on the engraftment susceptibility of intraportally injected HCC cells in a mouse model (15, 16). The anti-HCC activity of hepatic NK cells significantly decreased after partial hepatectomy, allowing intrahepatic metastasis growth in mice receiving HCC cells (16). Intravenous adoptive immunotherapy performed using activated NK cells extracted from normal livers markedly inhibited intrahepatic metastasis. NK-cell competence and ability to survey and eliminate *de novo* neoplastic cells may provide defense against both monocentric and multicentric recurrence.

The human liver contains an unusually high number of infiltrating immune cells; 30%–50% of lymphocytes are NK cells (2). Liver NK cells have unique properties, including TNF-related apoptosis-inducing ligand (TRAIL)–dependent cytotoxicity, high NKp46 and CD122 expression, and specific cytokine profiles (2, 3). TRAIL on NK cells binds to four receptors, including death-inducing receptors (DR4 and DR5) that signal apoptosis and decoy receptors (DcR1 and DcR2; refs. 17). Moderately/poorly differentiated HCC remarkably expresses DR4/DR5 but not DcR1/DcR2, increasing TRAIL-expressing NK cell–mediated cell killing susceptibility (2, 18). On the basis of those findings, we proposed a novel immunotherapy of intravenously injecting activated liver allograft–derived NK cells into liver transplant recipients to control HCC recurrence (19).

In addition to TRAIL, hepatic NK-cell roles in immune tumor surveillance are likely mediated by perforin, granzyme, and interferon-γ (20). Gene polymorphisms for KIR and its HLA ligands possibly contribute to the heterogeneous tumor-surveillance functions of NK cells and likely affect clinical HCC outcomes. Recently, a small cohort study of patients with HCV-related HCC who underwent curative treatment by either surgical resection or radiofrequency thermal ablation (RTA) showed that the compound KIR2DL2-C1 and KIR3DS1-Bw4T80 genotypes are associated with longer time to recurrence and worse OS, respectively (21). We also analyzed the impact of these genotypes in the present study, but did not observe consistent results (Table 1 and Supplementary Table S5). This discrepancy might be related to the fact that the time to recurrence was markedly longer in our study than that in the previous study (median time to recurrence = 29.7 vs. 17 months, respectively), which is likely due to the heterogeneity of the therapeutic modality used in the previous study (i.e., time to recurrence in patients treated with RTA was significantly shorter than that in patients treated by resection; ref. 21). Our propensity score–matched studies demonstrated that the presence of a single functional compound KIR-HLA genotype did not markedly affect HCC recurrence, but that compound KIR-HLA genotype multiplicity was associated with the HCC recurrence rate. Taken together, with this finding and the fact that the number and type of host MHC class I alleles quantitatively tune the responsiveness of individual NK-cell subsets expressing the corresponding KIR (14, 22), the effect of NK-cell licensing on HCC recurrence should be quantitative. This effect of NK-cell licensing on HCC recurrence reached statistical significance in the non–HCV-related cohort but not in the

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Table 1. Cumulative risk of recurrence and overall survival of patients with HCC according to clinicopathologic characteristics and compound KIR-HLA genotypes

	Total patients ($N = 170$)				HCV-related patients ($n = 97$)			
	Cumulative risk of recurrence		OS		Cumulative risk of recurrence		OS	
	P	HR (95% CI)	*P*	HR (95% CI)	*P*	HR (95% CI)	*P*	HR (95% CI)
Age (≤65 vs. >65 years)	0.745	NA	0.362	NA	0.626	NA	0.592	NA
Sex (male vs. female)	0.078	1.54 (0.99–2.53)	0.966	NA	0.554	NA	0.216	NA
Etiology (HBV vs. HCV vs. others)	0.448	NA	0.117	NA	NA	NA	NA	NA
Tumor number (≥2 vs. 1)	0.010	1.57 (1.06–2.31)	0.039	1.64 (1.11– 2.39)	0.088	1.56 (0.92–2.51)	0.604	NA
Maximum diameter (≤50 mm vs. >50 mm)	0.222	NA	0.511	NA	0.749	NA	0.313	NA
Histologic differentiation (G1+G2 vs. G3)	0.253	NA	0.498	NA	0.866	NA	0.909	NA
Vascular invasion	0.733	NA	0.851	NA	0.612	NA	0.454	NA
Satellite lesion	0.657	NA	0.743	NA	0.451	NA	0.486	NA
Surgical margin (<5 mm vs. ≥5 mm)	0.126	NA	0.162	NA	0.039	1.88 (0.98–3.36)	0.003	1.88 (0.97 – 3.37)
KIR2DL1-C2	0.113	NA	0.754	NA	0.189	NA	0.964	NA
KIR2DL2-C1	0.253	NA	0.945	NA	0.456	NA	0.745	NA
KIR2DL3-C1	0.793	NA	0.283	NA	0.694	NA	0.105	NA
KIR3DL1-Bw4	0.269	NA	0.359	NA	0.572	NA	0.935	NA
KIR3DL2-A3/11	0.585	NA	0.570	NA	0.845	NA	0.986	NA
Number of KIR-HLA genotypes (≥3 vs. ≤2)	0.016	0.61 (0.38–0.94)	0.224	NA	0.130	NA	0.735	NA

NOTE: Cumulative risk of recurrence and OS were compared by log-rank statistics for univariate analysis. Cox proportional hazards model was conducted for multivariate survival analysis. Only variables presenting $P < 0.1$ in the univariate analysis were included in the multivariate model. $P < 0.05$ was considered statistically significant.
Abbreviation: NA, not assessed.

HCV-related cohort, which might be explained by the fact that hepatic NK cells exhibited reduced cytotoxicity and TRAIL expression in patients with chronic HCV infection (23).

We demonstrated that patients at high risk of HCC recurrence after curative hepatectomy could be identified by KIR-HLA genotyping. Licensed NK cells generally have higher resting capacity for responses including interferon-γ production and cytotoxicity than unlicensed NK cells, but both NK-cell types are highly activated by *in vitro* stimuli (24). Therefore, therapeutic strategies manipulating NK-cell activity either *in vivo* or *in vitro* could compensate for genetic susceptibility to HCC recurrence. This concept might also be supported by a previous randomized trial demonstrating that adoptive immunotherapy with autologous lymphocytes activated *in vitro* with recombinant IL2 and anti-CD3 Abs decreased the frequency of recurrence after HCC curative resection (25).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: N. Tanimine, Y. Tanaka, H. Ohdan
Development of methodology: N. Tanimine, Y. Tanaka
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): N. Tanimine, T. Kobayashi, M. Imamura, H. Aikata, H. Ohdan
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): N. Tanimine, J. Tanaka, H. Ohdan
Writing, review, and/or revision of the manuscript: N. Tanimine, K. Chayama, H. Ohdan
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): N. Tanimine, D. Miki
Study supervision: H. Tashiro, J. Tanaka, H. Ohdan

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Werner Syndrome-specific induced pluripotent stem cells: recovery of telomere function by reprogramming

Akira Shimamoto[1]*, Koutaro Yokote[2] and Hidetoshi Tahara[1]

[1] *Department of Cellular and Molecular Biology, Graduate School of Biomedical and Health Sciences, Hiroshima University, Hiroshima, Japan*
[2] *Department of Clinical Cell Biology and Medicine, Graduate School of Medicine, Chiba University, Chiba, Japan*

Edited by:
Fumiaki Uchiumi, Tokyo University of Science, Japan

Reviewed by:
Antonella Sgura, Roma Tre University, Italy
In-Hyun Park, Yale University, USA

****Correspondence:***
Akira Shimamoto, Department of Cellular and Molecular Biology, Graduate School of Biomedical and Health Sciences, Hiroshima University, 1-2-3 Kasumi, Minami-ku, Hiroshima 734-8553, Japan
e-mail: shim@hiroshima-u.ac.jp

Werner syndrome (WS) is a rare human autosomal recessive premature aging disorder characterized by early onset of aging-associated diseases, chromosomal instability, and cancer predisposition. The function of the DNA helicase encoded by WRN, the gene responsible for WS, has been studied extensively. WRN helicase is involved in the maintenance of chromosome integrity through DNA replication, repair, and recombination by interacting with a variety of proteins associated with DNA repair and telomere maintenance. The accelerated aging associated with WS is reportedly caused by telomere dysfunction, and the underlying mechanism of the disease is yet to be elucidated. Although it was reported that the life expectancy for patients with WS has improved over the last two decades, definitive therapy for these patients has not seen much development. Severe symptoms of the disease, such as leg ulcers, cause a significant decline in the quality of life in patients with WS. Therefore, the establishment of new therapeutic strategies for the disease is of utmost importance. Induced pluripotent stem cells (iPSCs) can be established by the introduction of several pluripotency genes, including *Oct3/4*, *Sox2*, *Klf4*, and *c-myc* into differentiated cells. iPSCs have the potential to differentiate into a variety of cell types that constitute the human body, and possess infinite proliferative capacity. Recent studies have reported the generation of iPSCs from the cells of patients with WS, and they have concluded that reprogramming represses premature senescence phenotypes in these cells. In this review, we summarize the findings of WS patient-specific iPSCs (WS iPSCs) and focus on the roles of telomere and telomerase in the maintenance of these cells. Finally, we discuss the potential use of WS iPSCs for clinical applications.

Keywords: Werner syndrome (WS), accelerated aging, chromosomal instability, telomere dysfunction, induced pluripotent stem cells (iPSCs), reprogramming, telomerase, premature senescence phenotypes

INTRODUCTION

Werner syndrome (WS) is a rare human autosomal recessive disorder characterized by early onset of aging-associated diseases, chromosomal instability, and cancer predisposition (Goto, 1997, 2000). Fibroblasts from patients with WS exhibit premature replicative senescence (Salk et al., 1981b). *WRN*, the gene responsible for the disease, encodes a RecQ-type DNA helicase (Oshima et al., 1996; Yu et al., 1996; Goto et al., 1997; Matsumoto et al., 1997) that is involved in the maintenance of chromosome integrity during DNA replication, repair, and recombination (Shimamoto et al., 2004; Rossi et al., 2010).

WRN is a member of the RecQ helicase gene family, and other members of the family include BLM and RTS/RECQL4, which are mutated in Bloom syndrome (BS) and Rothmund–Thomson syndrome (RTS), respectively (Ellis et al., 1995; Kitao et al., 1999). BS and RTS, along with WS, are characterized by chromosomal instability, due to which RecQ helicases are considered to be the guardian angels of the genome (Shimamoto et al., 2004; Bohr, 2008). There are five members in the RecQ helicase gene family, including RECQL1 (Seki et al., 1994) and RECQL5 (Kitao et al., 1998; Shimamoto et al., 2000), the mutations of which have yet to be identified in human diseases.

Major clinical symptoms of WS include common age-associated diseases, such as insulin-resistant diabetes mellitus, and atherosclerosis. Recent advances in drug therapy for these diseases are available and are known to increase the lifespan of patients with WS. However, there is no effective therapy for intractable features, such as severe skin ulcers leading to a decrease in quality of life (QOL), which is a serious problem in patients with WS. Thus, there is an urgent need to develop a new treatment strategy for this syndrome. Regenerative medicine, such as autologous cell transplantation, could be considered as one of the therapeutic strategies for WS, and a potential choice is the use of patient-specific iPSCs.

Somatic cell reprogramming follows the introduction of several pluripotency genes, including *Oct3/4*, *Sox2*, *Klf4*, *c-myc*, *Nanog*, and *Lin-28*, into differentiated cells such as dermal fibroblasts, blood cells, and others (Takahashi and Yamanaka, 2006; Takahashi et al., 2007; Yu et al., 2007; Aoi et al., 2008; Stadtfeld and Hochedlinger, 2010; Okita and Yamanaka, 2011). During reprogramming, somatic cell-specific genes are suppressed, while embryonic stem cell (ESC)-specific pluripotency genes are induced, leading to the generation of induced pluripotent stem cells (iPSCs) with undifferentiated states and pluripotency

(Stadtfeld et al., 2008). Somatic cell reprogramming generates iPSCs characterized by pluripotency and infinite proliferative potential similar to the ESCs, and this technology opens up new possibilities for tailor-made regenerative medicine (Stadtfeld and Hochedlinger, 2010; Okita and Yamanaka, 2011).

Recently, two groups reported the generation of iPSCs from the cells of patients with WS and came to the similar conclusion that reprogramming repressed premature senescence phenotypes in WS cells (Cheung et al., 2014; Shimamoto et al., 2014). They demonstrated the successful reprogramming of cells from patients with WS into iPSCs with restored telomere function and stable karyotypes, suggesting that the induction of the gene encoding human telomerase reverse transcriptase (hTERT) during reprogramming suppresses telomere dysfunction in WS cells lacking WRN. In this review, we summarize the findings of WS patient-specific iPSCs (WS iPSCs) reported in the literature, and focus on the roles of telomere and telomerase in maintenance of these cells. We also review the recent progress in the clinical management of WS and explore stem cell therapy as a new strategy for WS treatment. WS iPSCs will provide opportunities not only for a better understanding of the pathogenic processes and modeling of the complex features of WS, but also for drug screening as well as the discovery and development of a new strategy for its treatment.

FUNCTION OF WRN HELICASE

Prolonged S-phase and reduction in frequency of DNA replication initiation observed in WS cells have implicated the role of WRN helicase in DNA replication (Hanaoka et al., 1983; Poot et al., 1992). The fact that WRN helicase interacts with several factors involved in DNA replication, including RPA, PCNA, FEN-1, and Topoisomerase I, supports this theory (**Figure 1**; Shimamoto et al., 2004; Rossi et al., 2010). WS cells are hypersensitive to a Topoisomerase I inhibitor, camptothecin (Okada et al., 1998; Poot et al., 1999), and WRN nuclear foci induced by the DNA damage caused by camptothecin are co-localized with RPA in the S-phase (Sakamoto et al., 2001). In addition, WRN helicase forms or unwinds the Holliday junction intermediate associated with a regressed replication fork (Sharma et al., 2004; Machwe et al., 2007). These observations suggest that the WRN helicase is involved in the re-initiation of a stalled replication fork. WS cells also show hypersensitivity to 4NQO that induces oxidative damage (Gebhart et al., 1988). Since accumulation of oxidative DNA damage is associated with aging, it is suggested that the WRN helicase is associated with one of the oxidative repair mechanisms, base excision repair (BER), and is known to interact with BER factors, polδ, polβ, PCNA, RPA, FEN-1, and PARP-1 (**Figure 1**; Rossi et al., 2010). Furthermore, the WRN helicase unwinds a BER substrate produced by uracil-DNA glycosylase and AP endonuclease (Ahn et al., 2004). It is also known that the helicase interacts with the double-strand break repair factors Ku, DNA-PKcs, and the Mre11-Rad50-Nbs1 complex, as well as the telomeric DNA protecting proteins, TRF1, TRF2, and POT1 (**Figure 1**; Shimamoto et al., 2004; Rossi et al., 2010). Additionally, Tahara et al. (1997) reported abnormal telomere dynamics in WS lymphoblastoid cell lines (LCLs) with weak or no telomerase activity. These findings suggest that the WRN helicase is involved in telomere metabolism. WRN helicase is shown to resolve Holliday junctions (Sharma et al., 2004), G-quadruplexes formed in telomere G-rich sequences (Mohaghegh et al., 2001), and higher-ordered DNA structures, such as the D-loop (Opresko et al., 2004). These DNA structures formed at telomere ends must be resolved during DNA replication to be accessible to DNA polymerases and telomerase, therefore, WRN helicase might function in the resolution of higher order structures in telomeric DNA.

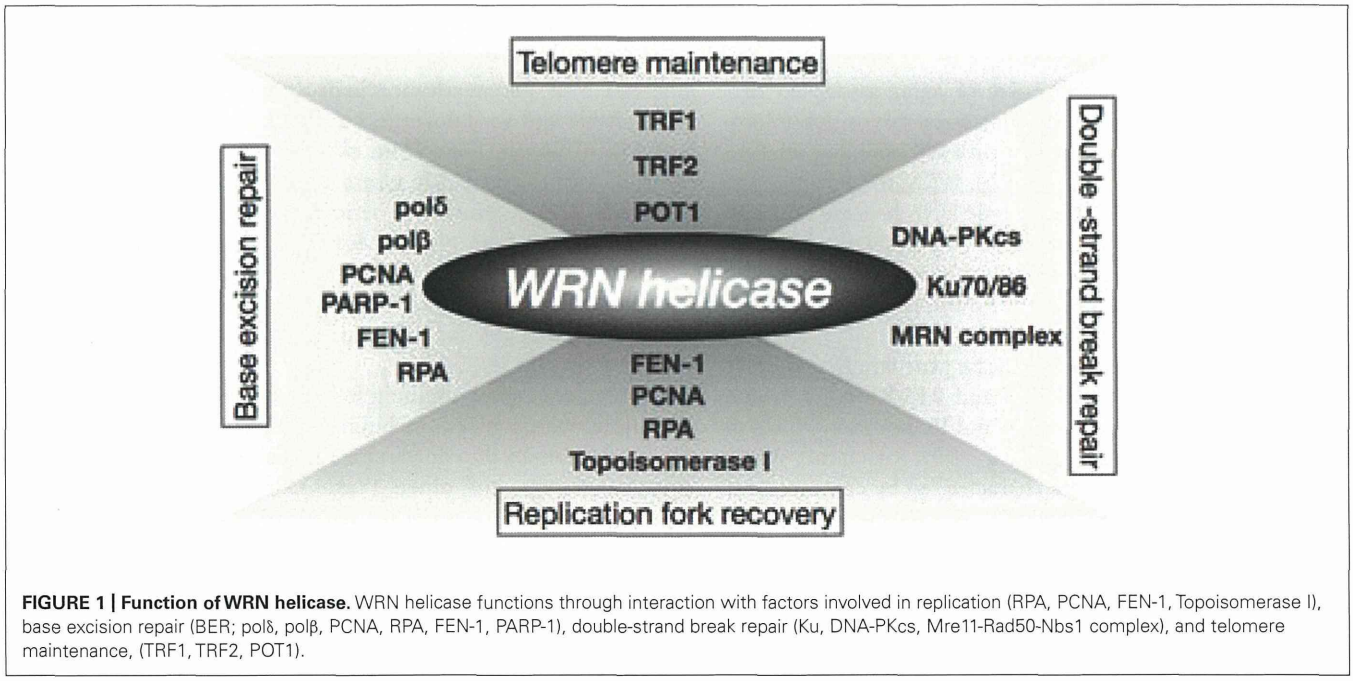

FIGURE 1 | Function of WRN helicase. WRN helicase functions through interaction with factors involved in replication (RPA, PCNA, FEN-1, Topoisomerase I), base excision repair (BER; polδ, polβ, PCNA, RPA, FEN-1, PARP-1), double-strand break repair (Ku, DNA-PKcs, Mre11-Rad50-Nbs1 complex), and telomere maintenance, (TRF1, TRF2, POT1).

ROLES OF TELOMERE IN REPLICATIVE LIFESPAN AND IMMORTALITY

Telomeres, the ends of linear chromosomes in eukaryotes, are ribonucleoprotein-containing specialized structures essential for the protection of chromosomes from a sensing mechanism of double-stranded DNA breaks (Chan and Blackburn, 2004). Mammalian telomeres are composed of TTAGGG repeat sequences, while their specific binding protein complex, shelterin, is composed of the six proteins TRF1, TRF2, RAP1, TIN2, POT1, and TPP1. The chromosome ends are capped by t-loop structures formed by the telomeric DNA and shelterin complex to protect them from DNA damage responses (Palm and de Lange, 2008). In normal human cells, progressive telomere shortening occurs with each successive cell division because of the "end replication problem," wherein regions of RNA primers involved in lagging strand DNA synthesis at most chromosome ends cannot be replaced with DNA during DNA replication (Harley et al., 1990; Levy et al., 1992). Most of the cells in the human body, such as terminally differentiated cells, have no detectable telomerase activity. Further, tissue stem cells such as hematopoietic stem cells (Vaziri et al., 1994; Allsopp et al., 2001, 2003), epidermal stem cells (Flores et al., 2005), and neural stem cells (Ferron et al., 2004) do not exhibit substantial telomerase activity that can add telomeric repeats sufficient to prevent their chromosomal ends from attrition with successive cell division, which is a major cause of human and other organismal aging (Blasco, 2007). On the other hand, germline stem cells and cancer cells express high levels of telomerase that maintains telomere length sufficient for their immortality (Flores et al., 2006). The human telomerase holoenzyme complex consists of a telomerase reverse transcriptase subunit, hTERT, and a template RNA, TERC, which are the basic components required for catalytic activity. (Egan and Collins, 2012) In addition, it also consists of other accessory proteins, including dyskerin, NHP2, NOP10, and NAF1 required for its assembly and stability (Egan and Collins, 2012). Introduction of hTERT is necessary and sufficient for the activation of telomerase in cells, as other components are already expressed in most normal cells and tissues (Nakayama et al., 1998; Chang et al., 2002). *hTERT* can elongate telomeres, extend the lifespan of normal cells, and immortalize cells such as dermal diploid fibroblasts (Bodnar et al., 1998; Vaziri and Benchimol, 1998; Jiang et al., 1999; Morales et al., 1999). Homologous recombination between telomeres, known as ALT (alternative lengthening of telomeres) is an alternative mechanism for the maintenance of telomere length, and has been observed in subsets of cancer cells, telomerase-deficient ESCs and iPSCs (Dunham et al., 2000; Niida et al., 2000; Wang et al., 2012). These findings indicate that the telomerase-dependent and -independent mechanisms of telomere maintenance are essential for cellular immortality.

WS FIBROBLASTS EXHIBIT PREMATURE REPLICATIVE SENESCENCE

Intrinsic DNA damage caused by the loss of WRN helicase could activate stress responses leading to cellular senescence. Senescence is defined as a state of permanent cell cycle arrest mediated by the p53-$p21^{Cip1/Waf1}$ and $p16^{INK4A}$-RB pathways. It is one of the tumor suppressor mechanisms exerted in cells that undergo replicative aging with telomere attrition, generation of reactive oxygen species, abnormal proliferation by oncogene activation, and DNA damage activated by DNA damaging agents such as ionizing radiation (Kuilman et al., 2010; Salama et al., 2014). Stress-associated p38 mitogen-activated protein kinase is constitutively activated in WS fibroblasts (Davis et al., 2005). Activation of p38 is known to mediate cellular senescence in the presence of elevated p21 levels (Haq et al., 2002; Iwasa et al., 2003), and p38 inhibitors can suppress premature senescence phenotypes of WS fibroblasts by reducing p21 expression (Davis et al., 2005). These observations indicate that p38 is a major mediator of the reduced replicative lifespan of WS fibroblasts. Meanwhile, activation of p38 also mediates induction of the senescence-associated secretory phenotype (SASP; Freund et al., 2011) that is the hallmark of aging. It is widely accepted that age-associated inflammatory responses contribute to human aging mechanisms (Goto, 2008). WS fibroblasts express inflammatory cytokines (Kumar et al., 1993), and WS is associated with inflammatory conditions responsible for common age-associated diseases, such as atherosclerosis, diabetes, and osteoporosis (Rubin et al., 1992; Murano et al., 1997; Yokote et al., 2004a; Davis and Kipling, 2006). Taken together, these findings suggest that premature replicative senescence with concomitant induction of p21 and SASP, mediated by the activation of p38, could be pathogenic hallmarks of WS.

TELOMERASE BYPASSES PREMATURE REPLICATIVE SENESCENCE IN WS FIBROBLASTS

As mentioned previously, WRN helicase might play an important role in telomere maintenance. This has been verified by Crabbe et al. (2004) wherein, defects in WRN helicase caused impairment of telomeric lagging-strand synthesis and accelerated telomere loss during DNA replication. Moreover, the telomere loss caused by mutation in the WRN gene involved telomere dysfunction such as chromosome end fusions (Crabbe et al., 2007). It is postulated that the absence of WRN causes stalled replication forks at the sites of unresolved G-quadruplexes at the lagging telomere, which would produce degradable substrates for factors involved in DNA repair and recombination, leading to accelerated telomere shortening (**Figures 2A,B**; Multani and Chang, 2007). More importantly, telomerase prevented sister telomere loss (STL) caused by defective telomeric lagging-strand synthesis and suppressed chromosome end fusions in *WRN*-deficient cells (Crabbe et al., 2004, 2007). These results demonstrate that telomerase can provide WS fibroblasts with a complementation effect by adding telomeric DNA "TTAGGG" to lagging telomeres that are lost during replication (**Figure 2C**). Since telomerase is also known to bypass premature replicative senescence in WS fibroblasts (Wyllie et al., 2000), it is suggested that premature senescence in WS cells might be caused by defects in telomeric lagging-strand synthesis followed by telomere loss during DNA replication (Sugimoto, 2014).

PATHOLOGY IN RECENT WS PATIENTS AND THEIR LIFESPAN

Although WS patients usually grow normally until they reach the late teens, they generally exhibit short stature during adulthood due to impaired maturation. In their 20s and 30s, WS patients start