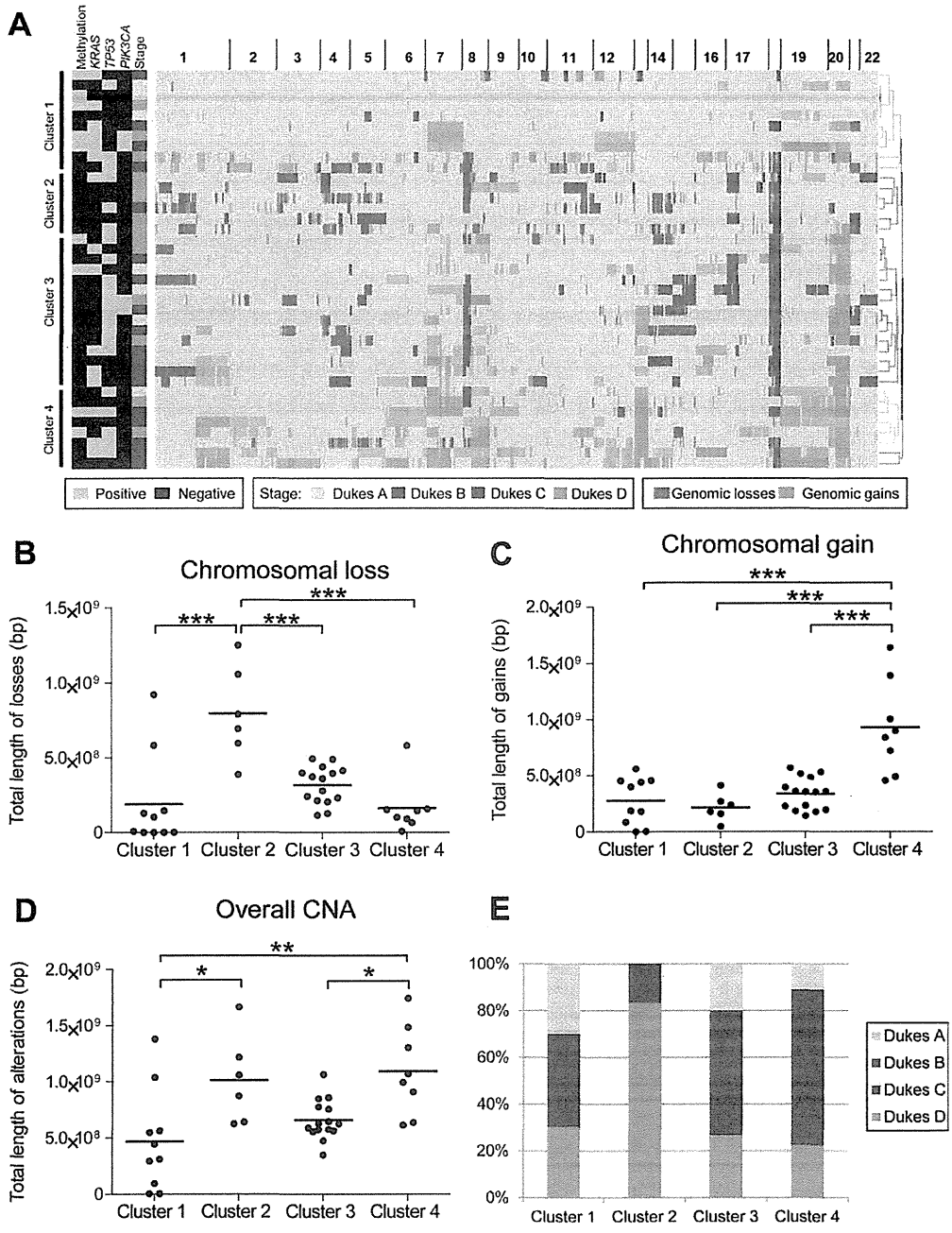

Figure 4. (A) Summarized results of unsupervised hierarchical analysis using the array-CGH data obtained from the 39 CRCs. Tumors were categorized into four clusters as indicated on the left. Tumors with frequent DNA methylation, *KRAS* mutation, *TP53* mutation, *PIK3CA* mutation, and Dukes' stages are also indicated on the left. (B–D) Total lengths of genomic losses and gains and overall CNAs in tumors within each cluster are shown. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. (E) Percentages of tumors at the respective Dukes' stages in each cluster are shown.

showed the greatest genomic gains. These results suggest that there is an inverse relationship between CIN and aberrant DNA methylation, and that there is a possible association between genomic losses and distant metastasis and between genomic gains and lymph node metastasis in CRC.

To determine whether a similar clustering pattern could be seen in an independent set of CRC samples, we carried out the same unsupervised

TABLE 2. Mean DNA Copy Number Alterations Within the Four Groups Obtained from the Unsupervised Clustering Analysis

Cluster	Cluster 1	Cluster 2	Cluster 3	Cluster 4	*P* value
Sample	10	6	15	8	
Age (mean ± SD)	66±14.7	69±11.2	69±10.1	63±15.2	
Sex					
Male	4	3	10	7	
Female	6	3	5	1	
Methylation	5/10*	0/6	2/15	2/8	
KRAS	6/10	1/6	5/15	3/8	
TP53	3/10	3/6	10/15	5/8	
PIK3CA	2/10	0/6	2/15	0/8	
18q loss	4/10*	6/6	15/15*	5/8	
20q gain	4/10*	3/6	14/15*	8/8	
18p loss	3/10*	4/6	13/15*	3/8	
8p loss	2/10*	4/6	11/15	4/8	
13q gain	1/10*	2/6	8/15	8/8*	
20p gain	2/10	0/6*	9/15	6/8*	
7p gain	4/10	1/6	5/15	6/8*	
7q gain	4/10	0/6*	7/15	5/8	
Loss (Mb)	189.4	795.8	318.5	162.6	<0.001
Gain (Mb)	277.4	216.3	339.1	930.3	0.001
Total CNA (Mb)	466.8	1012.1	657.6	1092.9	<0.001

Figures are the mean area of the CNAs per genome in each group, which were compared using one-way ANOVA. The total CNA is the sum of the losses and gains. Methylation, tumors with frequent DNA methylation.

*Significantly different from a random distribution, determined from the absolute value of the adjusted standardized residuals >1.96.

hierarchical clustering analysis using the publicly available data set (n = 121) reported by Nakao et al. (2004). We found that the CRC samples were subcategorized into four clusters, among which group 1 showed the highest levels of copy number gains, while group 3 showed the greatest genomic losses (Supporting Information Fig. 4).

DISCUSSION

CIN, which is inferred from DNA ploidy patterns, is the most common form of genomic instability in CRC and is defined by the presence of multiple structural or numerical chromosome changes (Rajagopalan and Lengauer, 2004; Grady and Carethers, 2008). Because mutations in mitotic checkpoint regulators are found in only a small population of CIN CRCs, the mechanism underlying CIN is largely unknown (Grady and Carethers, 2008). Moreover, no method for accurately evaluating the extent of CIN has yet been established. In this study, we used the total CNA length as a surrogate for CIN and analyzed its association with clinical and molecular variables. As described in an earlier study (Gaasenbeek et al., 2006), losses of heterozygosity (LOH) without accompanying copy number changes (e.g., somatic uniparental disomies) were not included as CNAs due to CGH array limitations. It is well established that estimates of CIN prevalence and mutation detection are influenced by the method used for analysis and the purity of the samples (Nakamura et al., 1994; Habano et al., 1996; Sugai et al., 2000; Cardoso et al., 2004; Issa, 2008). We therefore used a crypt isolation technique to avoid contamination by nontumorous cells. The high levels of marker gene methylation detected by quantitative pyrosequencing reflect the purity of the cancer cells in the isolated gland specimens and are indicative of the advantage of using the crypt isolation technique.

Several groups have reported an inverse relationship between CIMP and CIN (Goel et al., 2007; Cheng et al., 2008). Goel et al. (2007) found that CIN status measured as the LOH for eight microsatellite markers was inversely correlated with the methylation frequency at CIMP-related markers in sporadic CRCs, even those without MSI. In addition, Cheng et al. (2008) found that CIN measured as the number of chromosomal arms with gains/losses or LOH was inversely associated with CIMP status. In this study, we also observed an inverse relationship between concurrent methylation at multiple loci (which may represent CIMP) and CIN, although none of the tumors in this study exhibited *MLH1* methylation. Interestingly, we detected no significant difference in the magnitude of the chromosomal gains between tumors with frequent methylation and those without it. This suggests gains at certain genomic loci may be commonly involved in the pathogenesis of CRCs, irrespective of the methylation status. The presence of tumors with a high degree of chromosomal aberration in a subset of CIMP tumors may support this idea (Cheng et al., 2008).

There have been few studies in which the extent of chromosomal aberration during the progression of CRCs was analyzed as we have done in the present study. Hermsen et al. (2002) used conventional CGH to show that the progression of adenoma to carcinoma is associated with an increase in chromosomal aberration. A meta-analysis of 31 conventional CGH studies also showed that primary metastatic CRCs had significantly greater genomic alterations than non-metastatic CRCs (Diep et al., 2006). In addition, the results

of our array-CGH analysis suggest that accumulation of chromosomal losses may play an important role in the progression of CRCs from Dukes' A to Dukes' D. Unexpectedly, however, the overall level of CNAs was highest in Dukes' C tumors, with Dukes' D tumors showing somewhat less chromosomal alteration, which is probably attributable to the greater genomic gains in the Dukes' C group. Assuming that chromosomal gains are irreversible during CRC progression, at least a subset of Dukes' D tumors may have originated as Dukes' A or B tumors, not Dukes' C tumors with their larger chromosomal gains. Our results thus suggest that genomic losses may have a more significant impact on the metastatic properties of tumor cells than genomic gains, and that tumor cells that acquire losses at early stages in regions that are critical for metastasis may have a greater chance to metastasize to distant organs.

We also assessed the association between CNAs and tumor progression, including lymph node and distant metastasis. A previous meta-analysis of conventional CGH data suggests that losses at 4p are associated with the progression from Dukes' A tumors to Dukes' B–D tumors, and losses at 8p and gains at 7p and 17q correlate with liver metastasis (Diep et al., 2006). Our present findings that Dukes' D tumors frequently show losses at 4p and 8p and gains at 17q are consistent with those earlier findings. Importantly, our results seem to reflect the recent findings that losses at 4p and 8p are indicators of liver metastasis and a poor prognosis in CRC (Sheffer et al., 2009).

From an epigenetic viewpoint, Ju et al. (2011) reported that larger numbers of genes were methylated in stage I-III CRCs than in stage IV samples, and that CRCs at stages I-III exhibited methylation profiles that distinctly differed from the profiles of stage IV tumors. Together with the observation that even early stage tumors show intratumor heterogeneities, including ploidy pattern variation (Miyazaki et al., 1999), allelic imbalances (Boland et al., 1995; Sugai et al., 2005), and gene mutations (Baldus et al., 2010), tumor cells with metastatic potential may arise through early genetic and epigenetic lesions, as proposed in the "initiation" (Threadgill, 2005) or "parallel evolution" models (Gray, 2003).

In CRCs with distant metastasis, we frequently observed losses at 3q13.11, where *ACVR2B*, encoding activin A receptor type IIB, is located. Activins are growth and differentiation factors; they belong to the TGF-β superfamily and regulate cell differentiation, proliferation, and apoptosis in a variety of cancer cell types (Chen et al., 2006). Mutation of *ACVR2A*, which is 69% identical to *ACVR2B*, results in loss of its expression and is frequently found in MSI-positive CRCs (Jung et al., 2004). Furthermore, restoration of *ACVR2A* suppresses CRC cell growth, suggesting that it acts as a tumor suppressor (Jung et al., 2007). Together with an earlier report that expression of ACVR2B is weaker in CRC tissues than in normal colon mucosa (Babel et al., 2009), our findings suggest genomic loss of the *ACVR2B* locus may be involved in carcinogenesis and distant metastases in a subset of CRCs.

Consistent with the aforementioned meta-analysis of CGH data (Diep et al., 2006), we commonly observed genomic gains at 17q12, which encompasses a region encoding *ERBB2* and is frequently amplified and/or overexpressed in various types of cancer. One recent study showed that amplification of *ERBB2* leads to persistent activation of extracellular signal-regulated kinase 1/2 (ERK1/2) signaling, which in turn leads to resistance to treatment with cetuximab (Yonesaka et al., 2011). It has also been reported that the median overall survival was significantly longer for CRC patients without *ERBB2* amplification than for those with it (Yonesaka et al., 2011). Thus assessment of chromosomal aberrations at functionally important loci in CRCs could provide useful information for predicting distant metastasis and prognosis, as well as for the development of therapeutic strategies.

There have been a number of studies evaluating the utility of genomic instabilities, including CIN and CNAs, as prognostic markers in CRC (Pritchard and Grady, 2011). A recent meta-analysis confirmed that CIN (measured flow cytometrically as the presence of aneuploidy/polyploidy) is associated with an unfavorable prognosis (Walther et al., 2008). Our array-CGH analysis revealed an increase in copy number losses during the progression of tumor stage, suggesting that the total magnitude of genomic losses could potentially serve as a surrogate marker for genomic instabilities and a prognostic marker in CRC. Recently, Poulogiannis et al. (2010) carried out a hierarchical clustering analysis of array-CGH data obtained from 109 primary CRCs and showed that tumors could be categorized into four groups, in which tumors in groups I and II exhibited CNAs only infrequently, while those in groups III and IV exhibited an abundance of CNAs. CRCs in group I were characterized by a lack of CNAs and frequent MSI-positivity. This appears to be consistent with cluster 1 in our study, which was

enriched in tumors with frequent DNA methylation, and may suggest that the copy number profiles of CRCs with MSI are similar to those with frequent methylation but without *BRAF* mutation. They also reported that CRCs in group II, in which CNAs were somewhat more prevalent than in group I, were associated with a lack of lymph-node metastasis and a better prognosis, irrespective of MSI status (Poulogiannis et al., 2010). Thus group II may correspond to cluster 3 in our study, in which Dukes' B (lymph-node negative) tumors were significantly enriched. By contrast, most patients in our cluster 2 were staged as Dukes' D, with significantly greater genomic losses than tumors in other clusters. Furthermore, Dukes' C tumors were enriched in cluster 4, within which tumors exhibited the greatest genomic gains. Although the proportions of Dukes' C and D tumors in the study by Poulogiannis et al. differ from those in our study, the highest number of Dukes' D tumors were seen in group III and were characterized by significant genomic losses, while the majority of the Dukes' C tumors were in group IV and exhibited significant genomic gains. Although this distribution did not reach statistical significance, the tendency is consistent with our results. These findings suggest that cancers with gains are likely to metastasize to lymph nodes or unlikely to metastasize to distant sites. Thus a tumor's CNA pattern may define its metastatic behavior.

This study has several limitations, including a small sample size, the absence of tumors with *BRAF* mutation and/or *MLH1* methylation, and a lack of survival information; nonetheless, our results indicate several important findings. First, there is an inverse relationship between methylation status and the extent of the CNAs, particularly chromosomal losses. Second, tumor progression from Dukes' A to Dukes' C may be associated with the accumulation of CNAs, whereas a subset of Dukes' D tumors with smaller chromosomal gains may have been derived from Dukes' A or B tumors, but not Dukes' C tumors, which show significant genomic gains. Third, the different CNA patterns revealed by our hierarchical clustering analysis may be associated with different tumor behaviors, including local and distant metastasis, which suggests the presence of distinct molecular pathways in the development of CRC. Further study to clarify the differences between these subclasses will likely provide new insight into the molecular mechanisms that determine prognosis and the responses of CRCs to therapy.

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PLOS ONE

Forecasting Japan's Physician Shortage in 2035 as the First Full-Fledged Aged Society

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Abstract

Introduction: Japan is rapidly becoming a full-fledged aged society, and physician shortage is a significant concern. The Japanese government has increased the number of medical school enrollments since 2008, but some researchers warn that this increase could lead to physician surplus in the future. It is unknown how many physicians will be required to accommodate future healthcare needs.

Materials and Methods: We simulated changes in age/sex composition of the population, fatalities (the number of fatalities for the consecutive five years), and number of physicians from 2010 to 2035. Two indicators were defined: fatalities per physician and fatalities by physician working hour, based on the data of the working hours of physicians for each tuple of sex and age groups. We estimated the necessary number of physicians in 2035 and the number of new physicians to maintain the indicator levels in 2010.

Results: The number of physicians per 1,000 population is predicted to rise from 2·00 in 2010 to 3·14 in 2035. The number of physicians aged 60 years or older is expected to increase from 55,375 (20% of physicians) to 141,711 (36%). In 2010 and 2035, fatalities per physician were 23·1 and 24·0 for the total population, and 13·9 and 19·2 for 75 years or older, respectively. Fatalities per physician working hour are predicted to rise from 0·128 to 0·138. If working hours are limited to 48 hours per week in 2035, the number of fatalities per physician working hour is expected to be 0·196, and the number of new physicians must be increased by 53% over the current pace.

Discussion: The number of physicians per population continues to rise, but the estimated supply will not fulfill the demand for healthcare in the aging society. Strategies to increase the number of physicians and improve working conditions are urgently needed.

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Introduction

The population is aging rapidly in high-income countries. For example, Japan's total fertility rate was 1·37 in 2009, while the average life expectancy was 77·1 years for males and 84·4 years for females. People aged 65 or older accounted for 23·1% of the total population in Japan in 2010 [1]. This figure will increase to 38·7% in 2035 and represents the greatest proportion of elderly among high-income countries [1].

The number of Japanese physicians is low (2·15 per 1,000 population) compared with other high-income countries in the Organisation for Economic Co-operation and Development (OECD; mean = 3·00) [2]. Because population aging drives healthcare demand, [3] the physician shortage in Japan will likely become a bigger problem in the future.

The Japanese government strictly restricts physician supply, and Japanese medical schools impose a maxim level on medical school enrollment. As a result, a shortage of physicians, especially obstetricians and gynecologists, has emerged as a serious social issue [4–8]. Physicians' refusing to see high-risk patients and "bouncing" patients to other hospitals have attracted public concern [9]. Medical school enrollment has risen 16% (1,221 students) from 2008 to 2011 by the Japanese Government. The Japanese Government predicts that the number of physicians per 1,000 population will rise in the future. However, the Japan Medical Association and some researchers warn that the increase in physician numbers could lead to a surplus of physicians in the future and physician maldistribution should be solved [10–13].

However, these predictions are associated with several problems. First, overwork of physicians has not been considered. Japanese physicians work an average of 70·6 hours per week

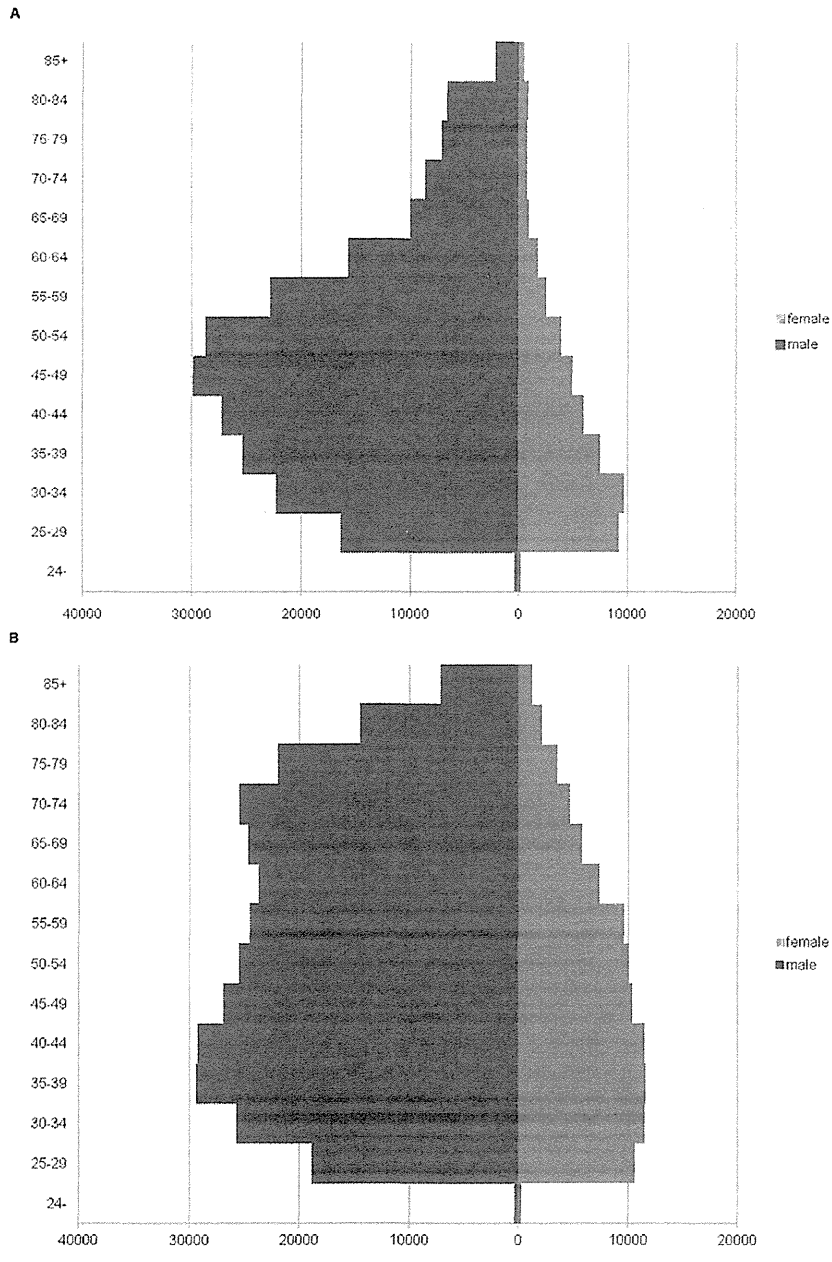

Figure 1. Physicians' population pyramids. Physicians' population pyramids for 2010 (panel A) and 2035 (panel B).
doi:10.1371/journal.pone.0050410.g001

(85 hours for those in their late 20 s and 48 hours for those in their 60 s) [14]. Japanese physicians have been reported to experience exhaustion, sudden death, and suicide from overwork [15]. It is important to restrict working time from the point of view of patient safety and physicians' health [16]. In Europe, working hours of junior doctors are limited to 48 hours per week by the EU Working Time Directive from 2009 [17]. In the United States, the Accreditation Council for Graduate Medical Education implemented duty hour limitations in 2003, contributing to improvement of patient safety, resident safety, and education [18].

Second, changes in sex and age composition have not been considered when discussing physicians' working hours. The number of practicing female physicians was 10,218 (9·3%) and 51,997 (18·1%) in 1965 and 2008, respectively [19]. Female doctors work fewer hours than their male counterparts (78 vs. 85 hours in their late 20 s and 40 vs. 48 hours in their 60 s) [14]. The supply of and demand for Japanese physicians have not been projected based on the actual physicians' workforce and patient/physician age structure.

An aging population is a common problem in high-income countries, and Japan will likely become a model of future

Table 1. Changes in simulated paramaters, 2010 and 2035.

	2010	2035	increase rate
The number of physicians per 1,000 population	2·00	3·14	57%
The number of populations	127,176,445	110,679,406	−13%
The total number of physicians	271,897	397,290	46%
The number of physicians aged 75 years or younger	254,126	347,103	37%
The number of physicians aged 75 years or older	17,771	50,187	182%
The number of physicians aged 60 years or younger	216,522	255,579	18%
The number of physicians aged 60 years or older	55,375	141,711	155%
The number of male physicians	222,784	297,483	34%
The number of female physicians	49,113	99,807	103%
practicing physicians' working hours per 1,000 population			
no limitation/current working hours	139·2	209·8	51%
limited to 60 hours/week		178·2	28%
limited to 48 hours/week		147·7	6%
The total number of fatalities for consecutive five years	5,881,151	8,336,263	42%
The number of fatalities for 75 years or older	3,529,540	6,650,448	88%
The number of fatalities for 74 years or younger	2,351,611	1,685,815	−28%
The number of fatalities per practicing physician	23·1	24·0	4%
fatalities per practicing physician for 75 years or older	13·9	19·2	38%
fatalities per practicing physician for 74 years or younger	9·2	4·8	−48%
The number of fatalities per practicing physician working hour			
no limitation/current working hours	0·128	0·138	8%
limited to 60 hours/week		0·162	27%
limited to 48 hours/week		0·196	53%

doi:10.1371/journal.pone.0050410.t001

healthcare systems. To forecast the balance between physician supply and healthcare needs, we simulated population, fatalities, and number of physicians in Japan from 2010 to 2035.

Materials and Methods

We simulated changes in population between 2010 and 2035 based on data provided by the National Institute of Population and Social Security [1]. The simulations were performed every 5 years from 2010 to 2035 for all of Japan and for each of the 47 prefectures. The number of fatalities for the consecutive five years (An estimate of the number of deaths in the next five years) was calculated based on the future life chart [1]. Let $x_{isj}(t)$ be the population of the ith prefecture ($i=1,...,47$), sex s ($s=1$ for male and $s=2$ for female), and jth the age group at year t. The population at year t+5 is predicted by

$$x_{isj}(t+5)=x_{isj}(t)\cdot\{d_{isj}(t:t+5)+m_{isj}(t:t+5)\}, \qquad (1)$$

where $d_{isj}(t:t+5)$ and $m_{isj}(t:t+5)$ are the rates of survival and migration during the 5 years from year t to t+5, respectively. We applied this concept to predict the distribution of the number of physicians at year t+5 from that at year t, denoted by $y_{isj}(t)$.

Simulation of the number of physicians by physician age/sex brackets was based on data from the 2008 National Survey of Physicians, Dentists, and Pharmacists [19]. We defined practicing physicians as physicians aged 75 years or younger. We calculated the number of practicing physicians per 1,000 population between 2010 and 2035. To estimate practicing physicians' working hours, we referred to data by gender and age provided by the Ministry of Health, Labour, and Welfare of Japan [14]. Male and female physicians worked 85 and 78 hours per week in their late 20 s, and 48 and 40 hours per week in their late 60 s, respectively [14]. In the simulation of the number of physicians, we need to consider the number of newly registered physicians. Let $n_{isj}(t)$ be the number of newly registered physicians of the ith prefecture, sex s, and jth the age group in year t. If we know $n_{isj}(t)$, Eq. (1) can be rewritten as

$$y_{isj}(t+5)=y_{isj}(t)\cdot\{d_{isj}(t:t+5)+m_{isj}(t:t+5)\}+n_{isj}(t+5).$$

In the simulation for physicians, age groups are defined as follows: The first age group includes physicians aged ≤24 years, and the remaining groups consist of physicians in 5-year intervals (e.g., age 25–29, 35–39, etc.).

We should note that no survey has been done to count the number of newly registered physicians for each tuple of prefecture, sex, and age group. Therefore, we estimated $n_{isj}(t)$ based on the data of the number of physicians in 2008 for each tuple provided by the Ministry of Health, Labour, and Welfare, of Japan [19]. First, we computed the basal level of $n_{isj}(t)$ that is independent of year by

$$\hat{n}_{isj}=max\left[y_{isj}(2008)-y_{isj-1}(2008)\cdot\{d_{isj-1}(2010:2015)+m_{isj-1}(2010:2015)\},0\right].$$

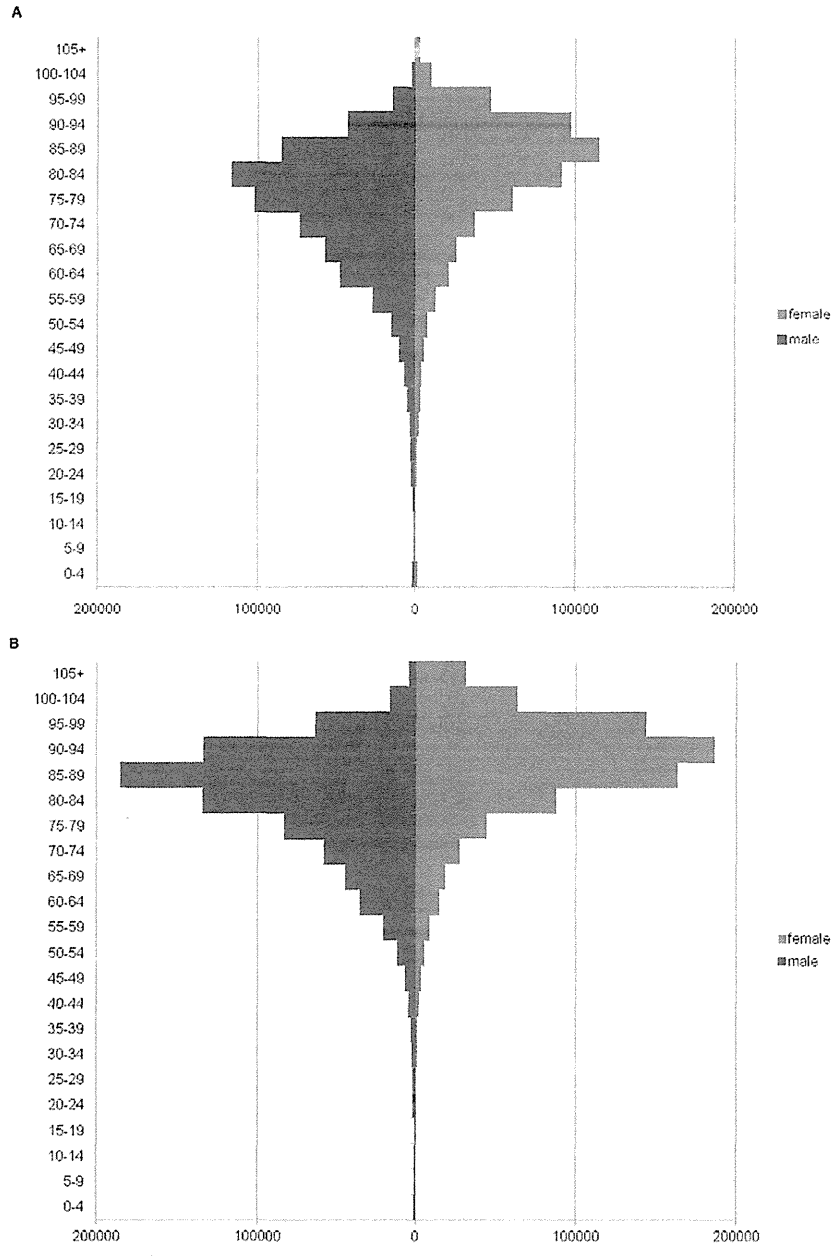

Figure 2. Fatality pyramids. Fatality pyramids for 2010 (panel A) and 2035 (panel B).
doi:10.1371/journal.pone.0050410.g002

We can consider that $\hat{n}_{isj}$ reflects the regular number of new medical school students in Japan around 2001, because a student who entered a medical school in 2002 is expected to have graduated in 2008. However, from 2008 to 2010, the regular number of the first-year medical students increased. To account for this effect, we multiply $\hat{n}_{isj}$ by the constant $c(t)$ and express the updated equation as

$$y_{isj}(t+5) = y_{isj}(t) \cdot \{d_{isj}(t : t+5) + m_{isj}(t : t+5)\} + c(t+5) \cdot \hat{n}_{isj}.$$

The constant $c(t)$ was determined by considering the actual regular numbers of first-year medical students in 2008, 2009, and 2010.

To determine working hours of physicians, we referred to data provided by the Ministry of Health, Labour and Welfare, Japan [13]. The data revealed that physicians in their late 20 s worked 85 hours per week. We modeled the working hours pre week, w_{sj}, for males by

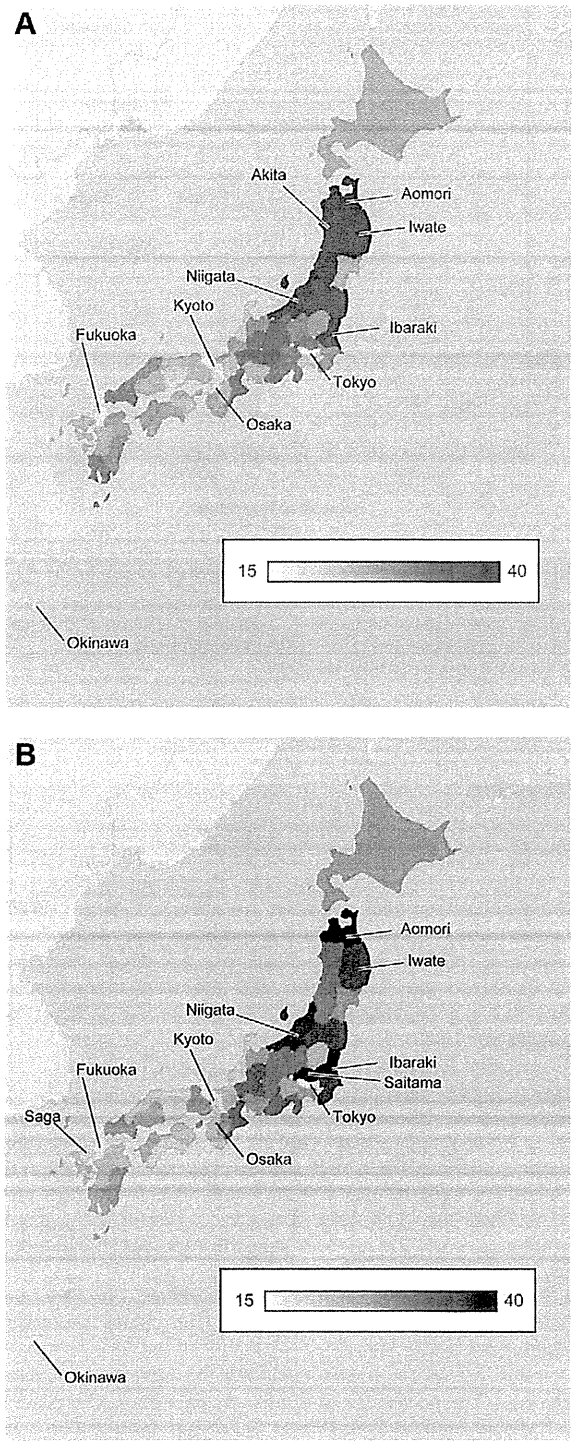

Figure 3. Number of fatalities per practicing physician for the entire population by prefecture. Values for 2010 are calculated and mapped (panel A). The indicator for all of Japan is 24·0. The best five prefectures are Tokyo (15·8), Okinawa (16·5), Kyoto (17·7), Fukuoka (18·2), and Osaka (19·1). The worst five prefectures are Aomori (35·2), Iwate (34·8), Akita (34·3), Niigata (34·2), and Ibaraki (32·7). Values for 2035 are calculated and mapped (panel B). The indicator for all of Japan is 23·1. The best five prefectures are Tokyo (15·7), Kyoto (17·3), Okinawa (17·3), Fukuoka (17·9) and Saga (20·0). The worst five prefectures are Saitama (38·2), Aomori (36·9), Ibraraki (36·1), Niigata (34·1), and Iwate (32·6).
doi:10.1371/journal.pone.0050410.g003

$$w_1^{male} = \alpha_m,$$
$$w_j^{male} = \{min(\alpha_m, \beta_m) - \alpha_m\} \cdot (j-2) + \alpha_m, \quad \text{for} \quad 2 \leq j \leq 9,$$
$$w_j^{male} = \beta_m, \quad \text{for} \quad 10 \leq j,$$

where the age groups corresponding to $j = 1$ and $j = 9$ are "≤24 years old" and "≥60and ≤64 years old," respectively. The working hours per week for females were similarly defined by

$$w_1^{female} = \alpha_f,$$
$$w_j^{female} = \{min(\alpha_f, \beta_f) - \alpha_f\} \cdot (j-2) + \alpha_f, \quad \text{for} \quad 2 \leq j \leq 11.$$

Note that the age group corresponding to $j = 11$ includes physicians whose age is ≥70 and ≤74 years. In the above model, the working hours per week follows the data of the Ministry of Health, Labour and Welfare, of Japan, by using $\alpha_m = 85$, $\beta_m = 48$, $\alpha_f = 78$, and $\beta_f = 40$. We controlled α_m and α_f in the simulations limiting working hours per week.

In the simulation of increasing the regular number of new students in medical schools, we defined the increasing rate as a; we assumed that the regular number of first-year medical students increases $(1+a)$ times that in 2010. We assumed that the policy for increasing regular numbers will be implemented for students who will enter medical schools in April 2013 and we thus revise a to zero and 3/5 times in 2015 and 2020, respectively. Note that we consider that the constant, a, is a positive value; however, this framework can represent a decrease in the regular number of new students and a more flexible determination of regular numbers by considering the year-dependent constant, $a(t)$.

We defined two indicators: the number of fatalities per practicing physician and the number of fatalities per practicing physician working hour. A total of 78·6% of Japanese people die in hospitals, [20] and the number of fatalities reflects actual healthcare demands. The indicators were estimated for the total population, for subjects aged 74 years or younger, and for subjects aged 75 years or older. The reason for setting a boundary at 75 years was that the average healthy life expectancy (HALE: the average number of years that a person can expect to live in full health by taking into account years lived in less than full health due to disease and/or injury) is 76 years (73 years for males and 78 years for females) in Japan, which is the highest in the world [21]. The percentage of persons who require long-term care increases explosively when they exceed 75 years of age [22].

We calculated serial changes in these indicators between 2010 and 2035, and estimated the required number of new physicians needed to maintain the levels of indicators through the study period. We further calculated serial changes in indicators among the 47 prefectures in Japan. Gray gradation maps of Japan were drawn using Google Visualization API (California, USA).

Results

Practicing physicians per 1,000 population

The number of practicing physicians per 1,000 population is predicted to increase from 2·00 in 2010 to 3·14 in 2035. The total number of practicing physicians is predicted to increase from 254,126 in 2010 to 347,103 in 2035, a 37% increase. The number of physicians aged 60 years or older is expected to increase from 55,375 (20% of physicians) in 2010 to 141,711 (36% of physicians) in 2035 (Table 1 and Figure 1).

The number of female physicians is expected to increase from 49,113 (15% of physicians) in 2010 to 99,807 (27% of physicians) in 2035.

Practicing physicians' working hours per 1,000 population

Practicing physicians' working hours per 1,000 population was predicted to increase from 139·2 hours in 2010 to 209·8 hours in 2035, a 51% increase (Table 1). If working hour regulations were implemented and physicians' working hours are limited to 60 hours and 48 hours per week in 2035, practicing physicians' working hours per 1,000 population were predicted to be 178·2 hours and 147·7 hours respectively, which correspond to a 28% increase and a 6% increase, respectively, compared with 2010.

Number of fatalities per practicing physician

The total number of fatalities for consecutive five years is predicted to increase from 5,881,151 in 2010 to 8,336,263 in 2035, a 42% increase (Figure 2 and Table 1). The number of fatalities for 75 years and older is predicted to increase from 3,529,540 in 2010 to 6,650,448 in 2035, an 88% increase. The number of fatalities for 74 years or younger is predicted to decrease from 2,351,611 in 2010 to 1,685,815 in 2035, a 28% decrease. The number of fatalities per practicing physician was predicted to increase from 23·1 to 24·0 (4% increase) between 2010 and 2035. The number of fatalities per practicing physician for 75 years or older is predicted to increase from 13·9 in 2010 to 19·2 in 2035, a 38% increase. The number of fatalities per practicing physician for 74 years or younger is predicted to decrease from 9·2 in 2010 to 4·8 in 2035, a 48% decrease.

Number of fatalities per practicing physician working hour

In 2010 and 2035, the numbers of fatalities per practicing physician working hour (100 hours) were predicted to be 0·128 and 0·138, respectively (Table 1 and S1). This indicator was predicted to increase by 8% in 2035 compared with 2010. However, if working hours were limited to 60 hours per week and 48 hours per week (as per EU limitations), these indicators were predicted to increase by 27% to 0·162 and 53% to 0·196, respectively.

Geographic distribution of physicians

Disparities by regions are anticipated in 2010 and 2035 (Figure 3 and S1). In 2010, the numbers of fatalities per practicing physician were lowest in Tokyo (15·8) and highest in Aomori (35·2). In 2035, they were lowest in Tokyo (15·7) and highest in Saitama (38·2) (Figure 3). In addition to the rural-urban inequalities, the indicators are predicted to be significantly high in 2035 in overpopulated areas surrounding Tokyo (Figure S2). When fatalities per practicing physician were compared between 2010 and 2035, the differential increase rates were correlated with prefectural populations (Figure 4).

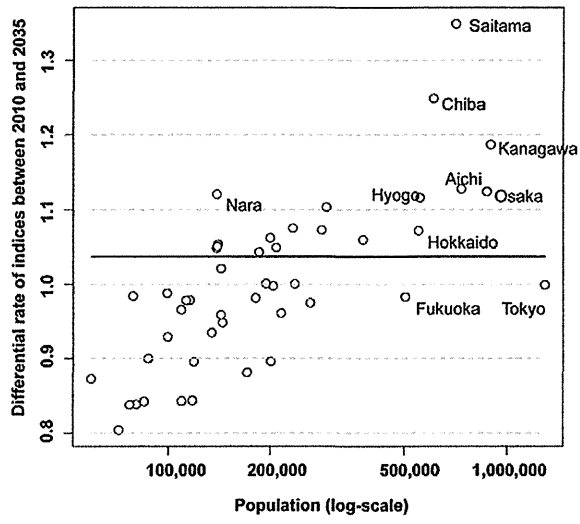

Figure 4. Correlation between prefectural populations and the differential increase in rates in the number of fatalities per physician for the entire population. When fatalities per practicing physician were compared between 2010 and 2035, the differential increase in rates was correlated with prefectural populations.
doi:10.1371/journal.pone.0050410.g004

Discussion

Our simulation showed future surges in healthcare demands due to the rapid aging of the Japanese population. Previous studies [11,12] have predicted that the number of physicians in Japan per 1,000 population in 2035 will reach the average level in OECD countries for the year 2010, which is consistent with our findings. However, physicians' working hours per population and the fatalities number per physician in 2035 suggests that physicians' supply will not be enhanced as expected by the increase in physician numbers.

One reason for overestimation is the aging of physicians. The aging of practicing physicians is of concern, with 35% of physicians being 60 years or older in 2035 compared with only 20% in 2010. Elderly physicians work much less compared with younger physicians (male and female physicians work 85 and 78 hours per week in their late 20 s, and 48 and 40 hours per week in their late 60 s) [14]. A marked aging of the general practitioners' workforce has been noted in Australia [23] and the Netherlands, [24] but the future impact of physicians' aging has not been evaluated fully. Changes in physicians' age structure should be considered when discussing the future physician workforce.

Another reason for overestimation is the increase in female physicians. The percentage of the female physicians who passed the National Medical Practitioners Qualifying Examination was 33·2% in 2011, and our simulation revealed that the percentage of female physicians is predicted to rise from 15% in 2010 to 27% in 2035. This trend is comparable to other countries [25–28]. Our simulation was based on a survey in 2011, and we did not consider future increases in female medical students. We might have underestimated the effect of increases in female physicians. To improve these situations, we should recognize that only 30% of female physicians are reinstated after retirement such as marriage

and childbirth [29]. Improvements in working conditions and the work environment will be necessary, especially for female physicians with small children [30,31].

We first simulated the future physicians' supply based on the physicians' actual working hours. Past projection of physician supply by the Japanese government was only based on the absolute number of physicians and did not focus on physicians' aging or the increase in female physicians. To restore the future "fatalities per physicians' working hours" indicator back to 2010 value, we showed that the number of new physicians needs to be increased by 8%. If working hours are limited to 48 hours per week, the increase rate jumps to 53%. The trends in implementation of work-time restrictions, based on patient safety and physicians' health, should be considered for future physicians' workforce prediction.

Medical innovation might have a huge impact on healthcare demand. The required number of physicians will increase in accordance with medical advancements [32]. Typical examples are gynecologists and pediatricians. In Japan, the number of live births decreased from 2,091,983 in 1973 to 1,071,304 in 2010, while a severe physician shortage is recently documented in pediatrics and obstetrics [14]. These findings suggest that medical renovation affects physicians' demands, and our simulation might underestimate future healthcare demands.

Our simulation predicted future geographic disparities of physicians. Previous reports showed that the geographic disparities between urban and rural areas has widened despite an increase in total physician number [12,33,34]. These findings were comparable to our study, and the difference in fatalities numbers between Tokyo (15·7 per physician) and Aomori (35·2 per physician) is predicted to remain in 2035. In addition to the rural-urban inequalities, the physician shortage will be significant in 2035 in overpopulated areas surrounding Tokyo. This is attributable to the mass-migration from rural areas to cities surrounding Tokyo, which occurred during Japan's economic boom after 1960. These migrants will age simultaneously, requiring medical care in future.

How can we tackle the future physician shortage? One solution is to establish new medical schools where physician shortages are severe. The United States, which will have 62,900 fewer doctors than needed in 2015, [35] are now planning to establish six new medical schools [36]. Some Japanese researchers proposed this solution to the Japanese government, but it will be difficult to achieve due to the strong opposition by the Japanese Medical Association [37]. Another solution is to allocate physicians to the shortage areas. Several prefectures are trying to settle residents to the areas with physician shortages by granting a scholarship to medical students, although these indirect measures play limited roles in ameliorating the physician shortage. Under the Japanese social system, neither government nor medical associations have the powers to force physicians to move to different areas.

This study has several limitations. First, we did not account for any possible medical advances. Our simulation might underestimate the necessary number of physicians. Second, the impacts of financial changes were not considered. Healthcare demand and supply are affected by economic status. For example, the Greek parliament approved a package of drastic healthcare cuts after the Greece debt crisis. Constant budget deficits in Japan might cause the same critical situation in the future. Finally, changes in the healthcare system might affect physician supply. The introduction of a new post-graduate clinical training system in 2004 impacted physicians' career paths and geographical distribution, [38] but this effect was not considered in this study.

In conclusion, a physician shortage for the aging society is predicted to be prominent in 2035. The change of age/sex structure of physicians has a negative effect on the workforce. Strategies to increase the number of physicians, improve working conditions, and solve poor distribution of physicians are urgently needed.

Supporting Information

Figure S1 Number of physicians per 1,000 population in Japan by prefecture. Values for 2010 are calculated and mapped (panel A, average: 2·00). The best five prefectures are Tokushima (2·61), Kyoto (2·60), Tokyo (2·56), Fukuoka (2·53), and Kochi (2·53). The worst five prefectures are Saitama (1·31), Ibaraki (1·45), Chiba (1·52), Aomori (1·61), and Niigata (1·63). Values for 2035 are calculated and mapped (panel B, average: 3·14). The best five prefectures are Kochi (4·33), Tokyo (4·30), Kyoto (4·11), Shimane (4·09), and Wakayama (4·02). The worst five prefectures are Saitama (1·97), Ibaraki (2·21), Chiba (2·35), Aomori (2·44), and Niigata (2·45).
(TIF)

Figure S2 Differential increase in the number of fatalities per physician for the entire population between 2015 and 2035. The indicator for all of Japan is 104%. The best five prefectures are Shimane (80) and Kochi, Fukui, Saga, Akita (84 each). The best five prefectures are Saitama (135), Chiba (125), Kanagawa (119), Aichi (113) and Osaka (112).
(TIF)

Table S1 Numbers of fatalities per practicing physician working hour in 2010 and 2035.
(XLS)

Author Contributions

Conceived and designed the experiments: KY SI RY SM MK. Performed the experiments: KY SI RY SM MK. Analyzed the data: KY SI RY SM MK. Contributed reagents/materials/analysis tools: KY SI RY SM MK. Wrote the paper: KY SI MK. Provided editorial advice: TM NM YK KI.

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Possible Involvement of Wnt11 in Colorectal Cancer Progression

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Our previous report revealed that the expression of Frizzled-7 (FZD7) in colorectal cancer (CRC) and its possible role in CRC progression. In this study we measured the expression levels of candidate FZD7 ligands, Wnt3 and Wnt11 in colon cancer cell lines ($n = 7$) and primary CRC tissues ($n = 133$) by quantitative RT-PCR. We also examined the functional effects of Wnt11 with the use of Wnt11 transfectants of colon cancer HCT-116 cells. Wnt11 transfectants showed the increased proliferation and migration/invasion activities compared to mock cells. Western blot analysis of transfectants revealed that phosphorylation of JNK and c-*jun* was increased after Wnt11 transfection. Wnt11 mRNA expression was significantly higher in the stage I, II, III, or IV tumor tissues than in non-tumor tissues (overall $P < 0.003$), while there was no significant difference in Wnt3 mRNA expression between tumor and non-tumor tissues. In addition, Wnt11 mRNA expression was significantly higher in patients with recurrence or death after surgery than in those with no recurrence (disease free) after surgery ($P = 0.018$). We also compared the expression levels of Wnt11 mRNA with those of FZD7 mRNA in the same CRC samples. Wnt11 mRNA expression was significantly higher in patients with higher FZD7 mRNA levels than in those with lower FZD7 mRNA levels ($P = 0.0005$). The expression levels of Wnt11 mRNA were correlated with those of FZD7 mRNA ($P < 0.0001$). These data suggest that Wnt11 may play an important role in CRC progression.

Key words: Wnt11; Frizzled-7; colorectal cancer; mRNA expression

INTRODUCTION

Wnt proteins are a family of secreted glycoproteins that serve as extracellular signaling molecules involved in cell proliferation, migration, and differentiation during embryonic development and lead to tumor formation when aberrantly expressed [1]. The Wnt signaling pathway is composed of canonical and non-canonical signals. Canonical Wnt signaling is mediated by frizzled transmembrane receptors and co-receptor low-density lipoprotein-related proteins. Their interaction promotes nuclear accumulation of β-catenin that associates with T-cell factor (Tcf)/lymphocyte enhancer transcription factors to activate target genes that are related to cell survival, proliferation, and invasion [2,3]. Non-canonical Wnt signaling is often attributed to one of two pathways termed the Wnt/Ca^{2+} pathway and Wnt/c-*jun* N-terminal kinase (JNK) (planar cell polarity) pathway [4,5]. In the Wnt/Ca^{2+} pathway, Wnt activates intracellular Ca^{2+} signaling, as well as Ca^{2+}-dependent protein kinases, such as calmodulin-dependent protein kinase II (CamKII) and protein kinase C (PKC). In the Wnt/JNK pathway, Wnt activates Rho family GTPases such as RhoA and Rac, as well as their downstream effectors, including Rho-associated kinase (ROCK) and JNK through Dishevelled (Del).

There are 19 Wnt proteins and 10 frizzled receptors (FZD) in vertebrates, suggesting that Wnt signaling specificity may be extremely complex. Wnt1, Wnt3, and Wnt8 activate canonical Wnt signaling, while Wnt5a and Wnt11 are thought to act primarily through non-canonical Wnt pathway [4]. However, recent studies suggest that individual Wnt proteins can activate either canonical or non-canonical Wnt signaling, depending on the type of cell [6,7], and some FZD can act in both canonical and non-canonical pathways [8]. Given that each member of the Wnt and FZD family is capable of binding to multiple partners, exploration of specific interactions of Wnts with FZDs could be an

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important issue to address in understanding Wnt signaling.

It is well known that the canonical Wnt signaling pathway is activated in most sporadic colorectal cancers (CRCs; up to 80%) and at least two-thirds of sporadic colorectal cancers acquire adenomatous polyposis coli (APC) gene mutations [9,10]. In addition, mutations of the β-catenin gene (CTNNB1) are also found but only in a small proportion of cases (up to 10%) [10]. These mutations were thought to cause constitutive downstream Wnt signaling independent of upstream signaling. Recently, however, involvement of upstream Wnt signaling regulation in CRCs has been reported, suggesting that Wnt signaling may be regulated in a quantitative manner at different levels [11,12].

In previous study, we found that Frizzled-7 (FZD7) was predominantly expressed in colon cancer cells and that FZD7 activated the canonical Wnt signaling in colon cancer cells despite the presence of APC or CTNNB1 mutation [13]. We also showed that the down-regulation of FZD7 expression decreased survival, invasion and metastatic capabilities of colon cancer cells through the canonical and non-canonical Wnt signaling pathways [14]. Furthermore, the expression levels of FZD7 mRNA in primary CRC were higher in tumor tissues than in non-tumor tissues and were higher in the recurrence or death group than in recurrence-free group [14]. These data suggest that FZD7 could play an important role in CRC progression, but the mechanism for activating FZD7 remains unknown.

It has recently been demonstrated that Wnt11 biochemically and functionally interacts with FZD7 to activate the non-canonical Wnt signaling pathway in vertebrates [15–17], and functional interaction between Wnt3 and FZD7 leads to activation of the canonical pathway in hepatocellular carcinoma cells [18]. Based on these findings we hypothesized that Wnt3 and Wnt11 may be candidate ligands for FZD7 in CRC and involved in its progression. To evaluate this possibility, we examined the expression levels of Wnt3 and Wnt11 mRNAs in colon cancer cell lines and primary CRC tissues. Furthermore, the functional effects of Wnt11 were investigated by using Wnt11-overexpressing colon cancer cells.

MATERIALS AND METHODS

Cell Culture

Human colon cancer cell lines DLD-1, HT-29, LoVo, SW-480, RKO and, HCT-116 were purchased from the American Type Culture Collection (ATCC, Manassas, VA). Human colon cancer Caco-2 cells were purchased from RIKEN BRC (Tsukuba, Japan). DLD-1 cells were cultured in RPMI 1640 medium (Gibco/Invitrogen, Carlsbad, CA) supplemented with 10% heat-inactivated FBS, 100 IU/mL penicillin, and 100 μg/mL streptomycin. HT-29 and HCT-116 cells were cultured in McCoy's 5A medium (Gibco) supplemented with 10% heat-inactivated FBS, 100 IU/mL penicillin, and 100 μg/mL streptomycin. LoVo cells were cultured in Ham's F-12K medium (Sigma, St. Louis, MO). SW-480 cells were cultured in Leibovitz's L-15 medium (Gibco) supplemented with 10% heat-inactivated FBS, 100 IU/mL penicillin, and 100 μg/mL streptomycin. RKO cells were cultured in minimum essential medium (Eagle; Sigma) supplemented with 10% heat-inactivated FBS, 100 IU/mL penicillin, 100 μg/mL streptomycin, 100 mM sodium pyruvate, and 10 mM non-essential amino acids. Caco-2 cells were cultured in minimum essential medium (Eagle; Sigma) supplemented with 20% heat-inactivated FBS, 100 IU/mL penicillin, and 100 μg/mL streptomycin.

Quantitative PCR

Total RNA was isolated from colon cancer cell lines and primary colorectal tumor and non-tumor tissues using the RNeasy Plus Mini Kit (Qiagen, Hilden, Germany) and All prep DNA/RNA Mini kit (Qiagen), respectively. The extracted total RNA was reverse-transcribed into single-stranded cDNA using a High-Capacity cDNA Archive Kit (Applied Biosystems, Warrington, UK). Real-time PCR was performed using first-strand cDNA with TaqMan® Gene Expression Master Mix (Applied Biosystems). The assay numbers for the endogenous control (β-actin) and target genes were as follows: 4326315E (β-actin); Hs00229135_m1 (Wnt3); Hs00182986_m1 (Wnt11); Hs00275833_s1 (FZD7). Quantitative PCR was performed on an ABI Prism 7900HT Sequence Detection System (Applied Biosystems). Quantitative PCR parameters for cycling were as follows: 50°C for 2 min hold, 95°C for 10 min 40 cycles of PCR at 95°C for 15 s and 60°C for 1 min. All reactions were carried out in a 20-μL reaction volume in triplicate. The mRNA expression level was determined using the $2^{-\Delta CT}$ method. For target gene assays, total RNA was isolated from colon cancer cell lines using the RNeasy Plus Mini Kit (Qiagen) after transfection. The extracted total RNA was reverse-transcribed into single-stranded cDNA using High-Capacity cDNA Archive Kit (Applied Biosystems). Real-time PCR was performed using first-strand cDNA with Power SYBR Green PCR Master Mix (Applied Biosystems). The primers used were as follows: c-Myc forward primer, 5′-tcaagaggtgccacgtctcc-3′; c-Myc reverse primer, 5′-tcttggcagcaggatagtcctt-3′; Cyclin D1 forward primer, 5′-ccgtccatgcggaagatc-3′; Cyclin D1 reverse primer, 5′-atggccagcgggaagac-3′; Survivin forward primer, 5′-cggttgcgctttcctttc-3′; Survivin reverse primer, 5′-tgttcttggctctttctctgtcc-3′; c-*jun*

forward primer, 5′-ggaaacgaccttctatgacgatg-3′; c-*jun* reverse primer, 5′-agggtcatgctctgtttcagg-3′; CD44 forward primer, 5′-tcatagaagggcacgtggtg-3′; CD44 reverse primer, 5′-tgggaggtgttggatgtgag-3′; CD44v6 forward primer, 5′-cccagaaggaacagtggtttg-3′; CD44v6 reverse primer, 5′-agctgtccctgttgtcgaatg-3′; CD44v8-9 forward primer, 5′-caggtttggtggaagatttgg-3′; CD44v8-9 reverse primer, 5′-tgtcagagtagaagttgttggatgg-3′; Met forward primer, 5′-caagaggagccccaccttatc-3′; Met reverse primer, 5′-ggcagtattcgggttgtaggag-3′; E-cadherin forward primer, 5′-ggccaggaaatcacatcctacac-3′; E-cadherin reverse primer, 5′-ttggcagtgtctctccaaatcc-3′; β-catenin forward primer, 5′-atgagactgctgatcttggacttg-3′, β-catenin reverse primer, 5′-gccatatccaccagagtgaaaag-3′; Vimentin forward primer, 5′-gagagaggaagccgaaaacacc-3′, Vimentin reverse primer, 5′-caaggtcaagacgtgccagag-3′; GAPDH forward primer, 5′-ccatgttcgtcatgggtgtg-3′; GAPDH reverse primer, 5′-ggtgctaagcagttggtggtg-3′. Quantitative PCR was performed on an ABI Prism 7900HT Sequence Detection System (Applied Biosystems). Quantitative PCR parameters for cycling were as follows: 95°C for 10 min 40 cycles of PCR at 95°C for 15 s, and 60°C for 1 min. All reactions were carried out in a 20-mL reaction volume in triplicate. The mRNA expression level was determined using the $2^{-\triangle CT}$ method.

Plasmid Construction and Transfection

The total RNA that was extracted from HCT-116 cells was reverse-transcribed into single-stranded cDNA using a High-Capacity cDNA Archive Kit (Applied Biosystems). The cDNA was subjected to PCR with KOD FX DNA polymerase (Toyobo, Tokyo, Japan) and with the primers, 5′-gcgcggtacctcctgggtgtgacc-3′ (forward; containing a KpnI restriction site at its 5′ end) and 5′-gtcggtttcctttgatgtcctg-3′ (reverse). The PCR products were purified by electrophoresis and digested with KpnI and EcoRI (site in Wnt11), and the released fragments were ligated into the multiple cloning site of a sequencing vector (pcDNA3.1[+]; Promega, Madison, WI). The resulting plasmids (pcDNA3.1-Wnt11) or empty vector plasmids were transfected into HCT116 cells using FuGENE HD (Roche Diagnostics, Basel, Switzerland) according to the manufacturer's instructions. To obtain HCT-116 cells stably trasfected with empty vector or pcDNA3.1-Wnt11, colonies of G418-resistant cells were selected and expanded for further characterization.

RNA Interference

Small-interfering RNAs were constructed in previously described [14]. Cells were transfected with scramble siRNA or FZD7_siRNA (siRNA8) by the Nucleofector system (Amaxa, Cologne, Germany).

Cell Proliferation Assay

We used the CellTiter 96® Aqueous One Solution Cell Proliferation Assay (Promega) according to the manufacturer's instruction. Briefly, cells (5×10^3) were seeded into 96-well plates and cell proliferation was measured with a CellTiter 96® Aqueous One Solution for 3 d. Results were presented as the average absorbance of three wells per experiment.

Cell Migration and Invasion Assay

For migration assays, we used the Transwell membrane filter inserts (8.0 mm pore size; Costar, Cambridge, MA). Cells were harvested and re-suspended in serum-free medium. Aliquots (1×10^4 cells) of the prepared cell suspension were added into the upper chamber, and the lower chamber was filled with 600 μL of culture media containing 5 μg/mL fibronectin (Sigma), as an adhesive substrate. Cells were incubated for 48 h or 72 h at 37°C in a 5% CO_2 tissue culture incubator. Migrated cells were stained with Diff-Quick solution (Fisher Scientific, Pittsburgh, PA).

Cell invasion assays were performed in parallel with the migration studies. Matrigel (1: 5; BD Biosciences, San Jose, CA) was added to Transwell membrane filter inserts (8.0 mm pore size; Costar) and incubated for 6 h at 37°C in a 5% CO_2 tissue culture incubator. Aliquots (1×10^5 cells) of the prepared cell suspension were added into the upper chamber, and the lower chamber was filled with 600 μL of culture media 5 μg/mL fibronectin (Sigma), as an adhesive substrate. Invasive cells were stained with Diff-Quick solution (Fisher Scientific).

Cells were counted with a microscope. The average number of cells in three fields per membrane was counted in triplicate.

Western Blot Analysis

Cells were washed in PBS and re-suspended in cold buffer containing 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM sodium orthovanadate, 0.1 mg/mL phenylmethylsulfonyl fluoride. Re-suspended cells were passed through the 21-gauge needle to shear the DNA and incubated for 30 min on ice followed by centrifugation at 10 000*g* for 10 min at 4°C. Total protein (5 μg) was analyzed by Western blotting using primary antibodies and anti-mouse and anti-rabbit IgG HRP-conjugated secondary antibodies (Dako, Glostrup, Denmark), and were visualized with Immuno-enhancer (Wako Pure Chemical Industries, Ltd., Osaka, Japan). The primary antibodies used were mouse anti-β-actin (Abcam, Cambridge, UK), anti-β-catenin, anti-JNK, anti-phospho-JNK, anti-c-*jun* and anti-phospho-c-*jun* (BD Biosciences) monoclonal antibodies, and rabbit anti-phospho-

β-catenin monoclonal antibody (Cell Signaling Technology). Quantification of relative band densities was performed by scanning densitometry using Image J software (National Institute of Health, Bethesda, MD).

Primary Colorectal Cancer Tissues and Non-Tumor Tissues

Wnt3 and Wnt11 mRNA expression was measured in colorectal tumors and non-tumor tissues obtained from 133 patients with CRC after receiving informed consent. The experimental protocol was approved by the institutional ethics committee of the Yamaguchi University Graduate School of Medicine, Ube, Japan. The mean age of patients was 67 ± 11 (mean ± SD) yr, and consisted of both men ($n = 70$) and women ($n = 63$). In all patients, the diagnosis of CRC was made on the basis of endoscopic and histological findings. A surgical operation was then carried out. The clinico-pathological characteristics of the patients after surgery were as described previously [14].

Statistical Analysis

Data analyses were performed using StatView 5.0, A Perfect Guide to Master (Nankodo Co., Ltd., Tokyo, Japan). The Mann–Whitney *U*-test, Student *t*-test, Wilcoxon signed rank test, two tailed Spearman's test or Kaplan–Meier analysis was used, as appropriate. A *P*-value less than 0.05 was considered to be statistically significant.

RESULTS

Wnt3 and Wnt11 mRNA Expression in Colon Cancer Cell Lines and Normal Colon Tissues

To evaluate Wnt3 and Wnt11 expression in colon cancer and normal colon tissues, quantitative measurement of their mRNAs with real-time PCR was performed for seven colon cancer cell lines (DLD-1, HT-29, LoVo, SW-480, RKO, HCT-116, and Caco-2) and five normal colon tissues. Although Wnt3 and Wnt11 mRNAs were detected in all the cell lines and normal colon tissues tested (Figure 1), expression levels of Wnt11 mRNA were relatively higher compared to those of Wnt3 mRNA.

Effect of Wnt11 on Cell Proliferation, Migration, Invasion, and Non-Canonical Wnt Signaling

Transfection of empty vector or pcDNA3.1-Wnt11 into HCT-116 cells that exhibited relatively lower level of Wnt11 expression in seven cell lines tested was performed. Wnt11 mRNA expression was significantly increased in the five pcDNA3.1-Wnt11 transfected clones (clone3, 33, 37, 48, 88) compared to untreated HCT-116 cells or cells transfected with empty vector (mock) (Figure 2A), whereas FZD7 mRNA expression was not changed

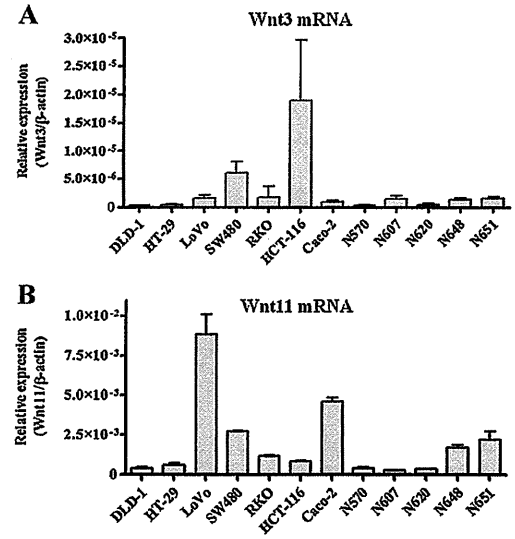

Figure 1. Wnt3 (A) and Wnt11 (B) mRNA levels detected by real-time RT-PCR in colon cancer cell lines and five normal colon tissues. β-actin was used as an endogenous control. Data are presented as mean values and ±SD for three independent experiments.

(Figure 2B). Among them, clone3 (WC-H) with relatively higher expression of Wnt11 and clone48 (WC-L) with relatively lower expression of Wnt11 were selected and used for the following studies.

We examined whether Wnt11 affects proliferation, migration, and invasion activities of HCT-116 cells. As shown in Figure 2C, cell proliferation was modestly but significantly increased in trasnsfectants (WC-H > WC-L) compared with mock cells at day 3 ($P < 0.0001$ for WC-H, $P = 0.0061$ for WC-L by Student *t*-test) and day 4 ($P = 0.037$ for WC-H, $P = 0.046$ for WC-L by Student *t*-test). Invasion and migration activities were also significantly increased in transfectants compared to mock, although there was no significant difference between WC-L and mock (Figure 2D and E) ($P = 0.0014$ or 0.030, respectively, in Figure 2D and E by Student *t*-test).

We then evaluated whether Wnt11 induces activation of Wnt/JNK pathway by Western blot analysis. The band intensities of phospho-JNK and phospho-c-*jun* were increased in Wnt11 transfectans (WC-H and WC-L cells) compared with mock, whereas there was no change in the band intensities of β-catenin and phospho-β-catenin (Figure 3A–D). As Wnt11 does not appear to stabilize β-catenin and is frequently found to inhibit canonical Wnt/β-catenin signaling pathway [19–21], the effect of Wnt11 transfection on the expression level of several canonical and non-canonical signaling target genes was examined.

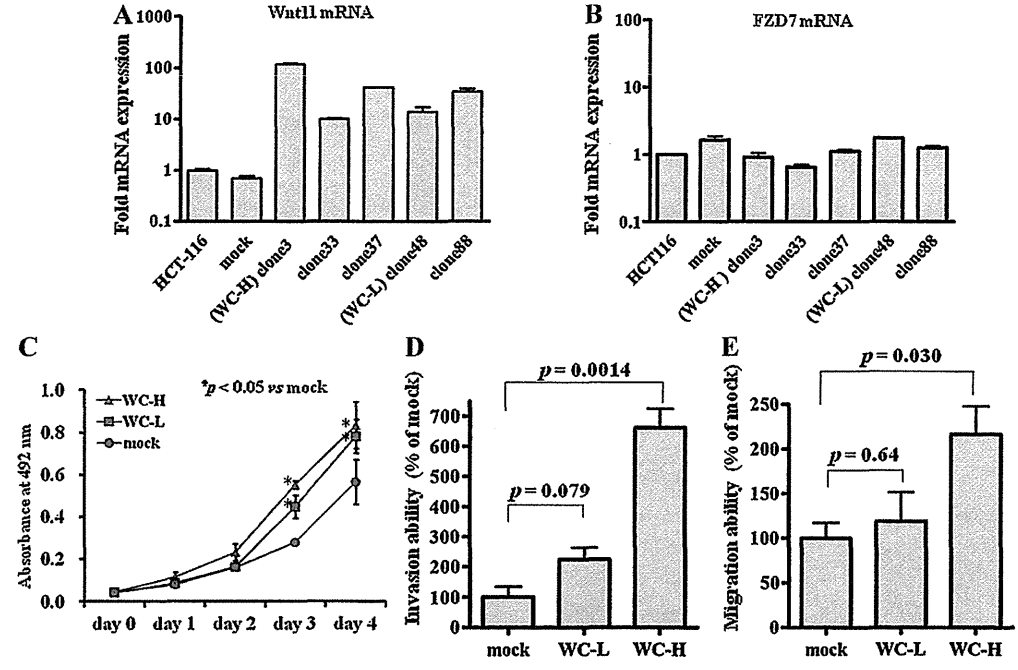

Figure 2. Preparation of Wnt11 transfectants and effect of Wnt11 on proliferation, migration and invasion of colon cancer HCT-116 cells. Real-time PCR analysis of Wnt11 (A) and FZD7 (B) mRNA expression in HCT-116 cells stably transfected with the expression vector harboring Wnt11 cDNA. Data of five clones are presented. The values were normalized for β-actin and compared to mock. (C) Effect of Wnt11 on the in vitro growth of HCT-116 cells. Cells transfected with an empty vector (mock) were used as a control. Cell growth of two transfectant clones (WC-L and WC-H) or mock for 4 d was determined by absorbance at 492 nm. This assay was performed as triplicates for each time point and repeated three times. (*$P < 0.05$ vs. mock by Student t-test). (D, E) Effect of Wnt11 on migration and invasion of HCT-116 cells. An aliquot of the prepared cell suspension was added into the upper chamber. The lower chamber was filled with culture media containing fibronectin and cultured for 48 h. Invaded or migrated cells were stained and the average number of cells in three fields per membrane was counted in triplicate and compared to mock. Data are presented as mean values and ±SD.

As shown in Figure 3E, the expression level of c-*jun*, CD44, CD44v6, CD44v8-9, and E-cadherin was clearly increased after Wnt11 transfection, although further studies are required to clarify the signaling pathways.

Effect of FZD7 siRNA Transfer on Invasion and Migration Activities of Wnt11 Transfectants

To evaluate the involvement of FZD7 in the increased invasion and migration activities of Wnt11-transfected HCT-116 cells, FZD7 siRNA [14] was transiently transferred into Wnt11 transfectans (WC-H and WC-L cells). As shown in Figure 4A and B, the expression level of FZD7 mRNA was clearly decreased after transfection of FZD7 siRNA whereas that of Wnt11 was not changed. Migration activity of Wnt11 transfectant WC-H cells was significantly decreased after FZD7 siRNA transfer, but that of WC-L cells was not affected (Figure 4E and F). In contrast, no significant effect of FZD7 siRNA on invasion ability was observed in both WC-H and WC-L cells (Figure 4C and D).

Wnt3 and Wnt11 mRNA Expression in Primary Colorectal Tumor Tissues and Non-Tumor Tissues

Using the same tissue samples as used in our previous report [14] the expression levels of Wnt3 and Wnt11 mRNAs in 133 primary CRC and 41 non-tumor tissues were examined. Similar to data from colon cancer cell lines (Figure 1A), the expression levels of Wnt3 mRNA were generally low and was detectable in only 29% (39/133) of the CRC tissue samples (Figure 5A). There was no significant difference in Wnt3 mRNA expression between each stage of tumor tissues and non-tumor tissues (Figure 5A). On the other hand, the expression levels of Wnt11 mRNA were relatively higher than those of Wnt3 mRNA and significantly higher in stage I, II, III, or IV tumor tissues than in non-tumor tissues ($P = 0.0022$, $P < 0.0001$, $P < 0.0001$, or $P < 0.0001$, respectively, by the Mann–Whitney

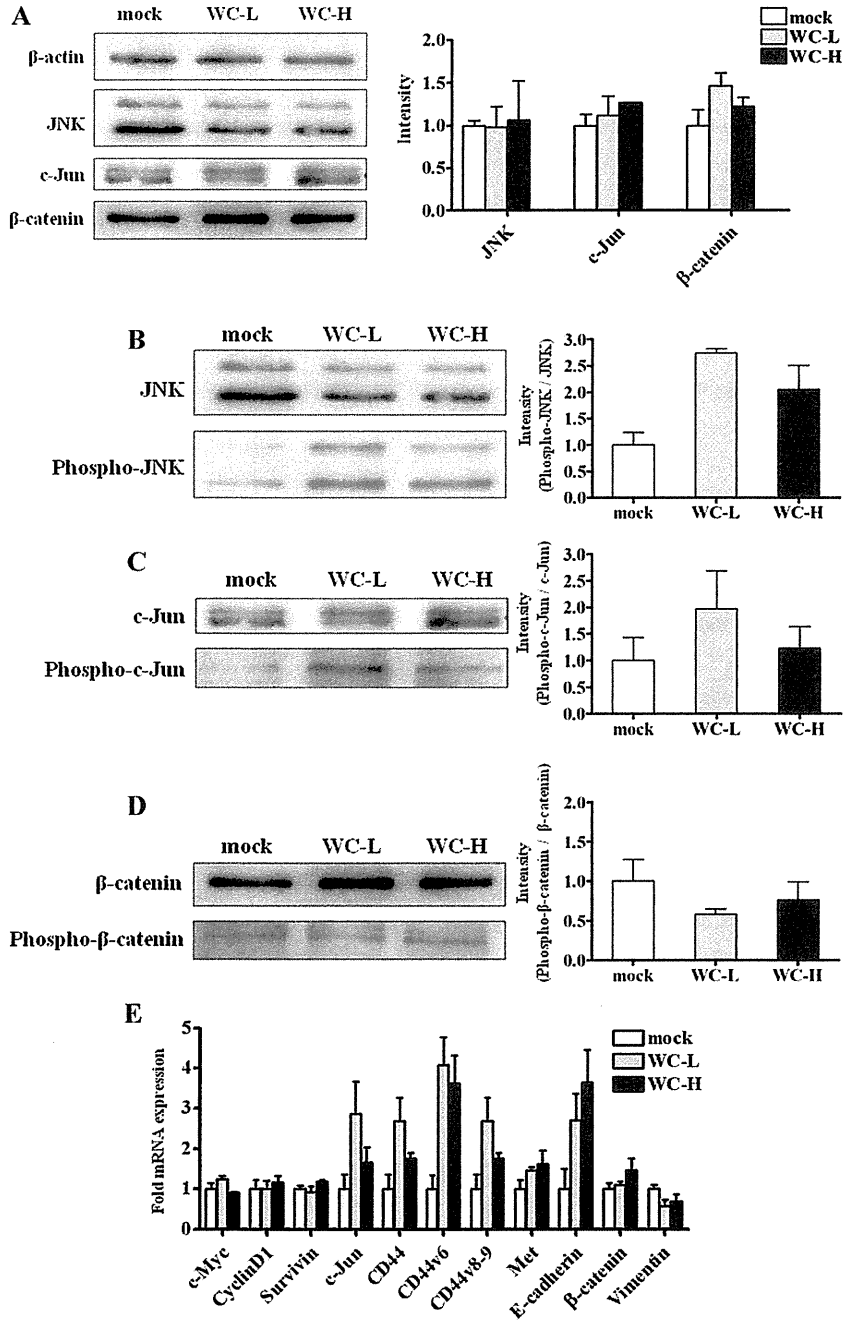

Figure 3. (A–D) Effect of Wnt11 on expression or phosphorylation of Wnt signaling proteins. Cytosolic protein (5 μg) in stable Wnt11 transfectants (WC-L and WC-H) or mock was subjected to Western blot analysis. β-actin was used as a loading control. The bar graphs depict the results of densitometric analysis from the Western blots. Ratio of band intensity indicates the ratio of band intensity of tested protein to that of β-actin (A), JNK (B), *c-jun* (C), or β-catenin (D). Data are presented as mean values and ±SD. (E) Real-Time PCR analysis of expression of Wnt target genes in mock, WC-L or WC-H cells. Total RNA were reverse-transcribed and amplified with primers specific for each indicated Wnt target as described in the Materials and Methods section.

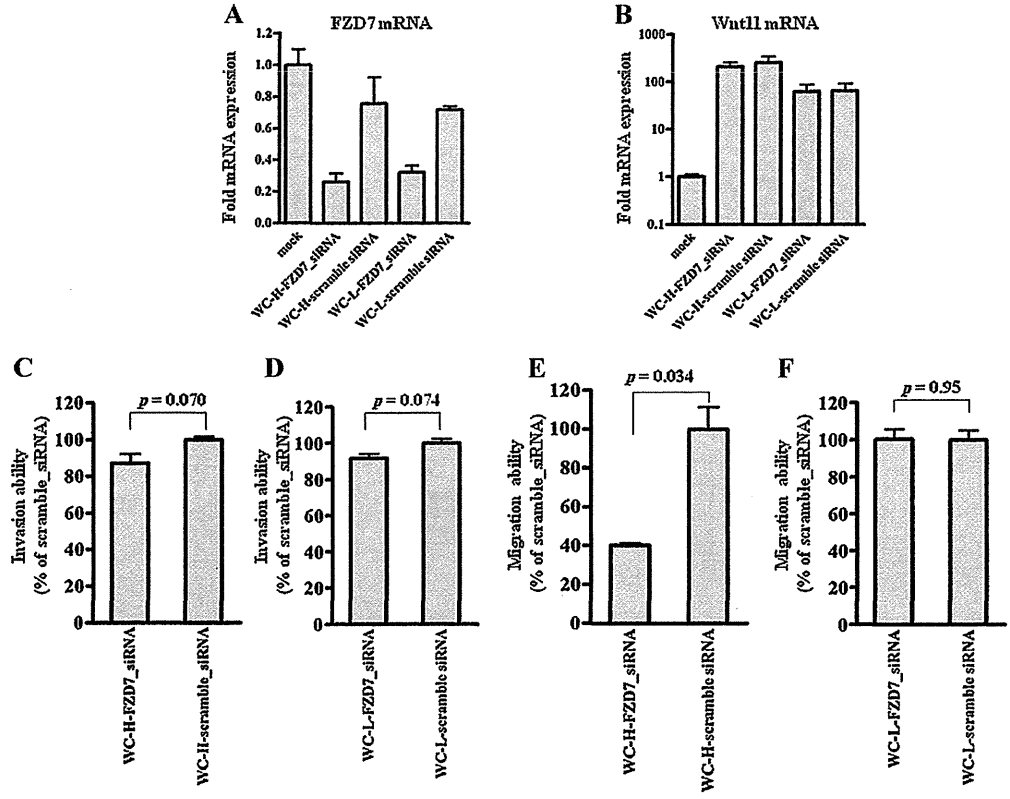

Figure 4. Effect of FZD7_siRNA on cell invasion and migration. Real-time PCR analysis of FZD7 (A) and Wnt11 (B) mRNA expression in WC-H or WC-L cells transfected with siRNA expression vectors harboring siRNA against FZD7 or scramble siRNA as previously described [14]. At 48 h after transfection, total RNAs were reverse-transcribed and the levels of mRNA expression of FZD7 and Wnt11were measured by real-time PCR. The values were normalized for β-actin and compared to mock. WC-H (C, E) and WC-L (D, F) were transiently transfected with FZD7_siRNA or scramble siRNA control. At 48 h after transfection, an aliquot of the prepared cell suspension was added into the upper chamber. The lower chamber was filled with culture media containing fibronectin and cultured for 72 h. Invaded (C, D) or migrated (E, F) cells were stained and the average number of cells in three fields per membrane was counted in triplicate and compared to scramble siRNA control. Data are presented as mean values and ±SD.

U-test; Figure 5B). To confirm the higher expression of Wnt11 mRNA in tumor tissues, we compared the Wnt11 mRNA level in 41 pairs of human CRC tissues and corresponding non-tumor tissues. Increased expression of Wnt11 mRNA in CRC tissues was observed in 32 out of 41 pairs (78%) ($P < 0.0001$ by Wilcoxon signed rank test; Figure 5C). There was no association of Wnt3 or Wnt11 mRNA expression with age, sex, tumor grade, lymph node metastasis, distant metastasis, histological type in univariate analysis (data not shown).

We next investigated the relationship of Wnt11 mRNA levels with prognosis. Follow-up information regarding survival was available for 120 patients as previously reported [14], and there was no significant difference in clinico-pathological characteristics between this patient group ($n = 120$) and total patients ($n = 133$) [14]. The expression level of Wnt11 mRNA was significantly higher in patients with recurrence or death after surgery compared to those with no recurrence (disease free) after surgery ($P = 0.018$ by Mann–Whitney *U*-test; Figure 6). However, there was no significant association of mRNA level with overall survival (data not shown).

Relationship Between Wnt11 and FZD7 mRNA Expression Levels

We previously showed that the expression level of FZD7 mRNA was higher in CRC tissues than in non-tumor tissues and was higher in patients with recurrence or death than in those without recurrence [14]. We therefore compared the expression levels of Wnt11 mRNA with those of FZD7 in the same CRC samples. Wnt11 mRNA expression was