result of multiple processes, including regulation of apoptosis and efflux of platinum drugs. Thus, other unknown chemoresistant mechanisms may be induced by overexpression of Anx A4. Because overexpression of Anx A4 has been reported in several other types of clinically important cancers, such as rectal, renal, lung and pancreatic cancer,[19–23] targeting Anx A4 may lead to the development of an effective therapy for overcoming chemoresistance in more types of cancer.

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Original Article

Evaluation of a Free-Coupon Program for Cervical Cancer Screening Among the Young: A Nationally Funded Program Conducted by a Local Government in Japan

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ABSTRACT

Background: Finding ways to improve the cervical cancer screening rates among young women has been seen as a critical national health problem in many countries, including Japan. The aim of the present study was to evaluate the effects of a free-coupon program for cervical cancer screening conducted by a local government under financial support from the Japanese national government.

Methods: The personal cervical cancer screening information was analyzed for all female residents of Toyonaka City, including any past screening history and clinical results since the year 2009, when a free-coupon program for screening was started. These results were compared to results from 2008, prior to implementation of the free-coupon screening program.

Results: The screening rates of women eligible for the free-coupon peaked dramatically compared to women of similar age who paid for their screening; however, the rates for the ineligible-age population also increased significantly in parallel to those in the free-coupon program, possibly by indirect peer and publicity effects. In women aged 20 to 25 years, the consecutive screening rate after a free-coupon screening was significantly lower than for those women who received a regular residential screening. After a free-coupon screening, the rate for participating in consecutive screenings depended significantly on the institution where the participant received her first screening test.

Conclusions: These results suggest that, for a generation of young women 20–25 years of age, a free-coupon program for cervical cancer screening was effective in increasing the first-time participation rate for screening; however, the increase in first-time participation did not lead to the expected increase in consecutive screenings.

Key words: cervical cancer screening; free-coupon; screening rate; consecutive screening

INTRODUCTION

Cancer of the cervix is the second most common cancer in women worldwide, with about 500 000 new cases and 250 000 deaths each year.[1] Almost 80% of cases occur in low-income countries.[1] Although a vaccine against the human papillomavirus (HPV) effectively prevents human papillomavirus infection and thus reduces the risk of cervical cancer by around 70%,[2] about 30% will still develop cervical cancer.

In some countries, including the United States and the United Kingdom, the cervical cancer screening rate is roughly 80%; however, in Japan it is only 25%.[3] Of particular concern, the screening rate for women aged 20–29 years is less than 10%.[4] Further, the incidence of cervical cancer among this 20- to 29-year age group has recently been increasing dramatically.[5] Finding ways to improve the screening rates among this younger generation has been seen as a critical national health problem.

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In Japan, it is recommended that women start receiving cervical cancer screening at age 20, to be repeated every 2 years. Even if women skip a screening test in the appropriate second year, they can still undergo a screening test the following year. The local government covers part of the screening costs, and the participant pays the rest, which usually amounts to ¥500 to ¥2000 (approximately $5 to $20 in United States' dollars [USD]). In 2009, a free-coupon program for screening for cervical and breast cancers was introduced in Japan as a national policy. In this program, a coupon or voucher for a free cervical cancer screening was sent by mail to women aged 20, 25, 30, 35, and 40. The program costs were covered by local governments, with financial support from the national government. Because this free-coupon program was terminated at the end of 2013, all citizens aged 20–44 years in Toyonaka had received a free-coupon only once between 2009 and 2013. A woman aged 20 in 2009, for example, would have received a free-coupon screening in 2009 and undergone a regular screening in 2011 and 2013.

There is an evidence gap as to whether removal of out-of-pocket costs and receipt of an individual invitation letter would be effective for increasing the cervical cancer screening rate, especially in Asia.[6–9] However, the reason for this inconsistency is unclear.

Toyonaka is an urban city located in Osaka prefecture. In October 2013, Toyonaka had an area of 38.6 km^2 and a population of 394 004. Toyonaka is officially acknowledged by the national government of Japan as a core city. In the present study, we evaluated the effectiveness of the free-coupon program in improving cervical cancer screening rates among the younger population of Toyonaka.

It was recently reported that removal of out-of-pocket costs for cervical cancer screening was an effective means of increasing the screening attendance of eligible women.[6] In the present study, we analyzed for the first time the effects of the free-coupon on the screening rate not only for the eligible women but also for the coupon-ineligible women, as well as the results of the screening tests and the consecutive screening rates following the free-coupon screening.

MATERIALS AND METHODS

The personal screening information of all female residents aged 20–49 in Toyonaka, including screening history and test results since 2009 (when the registration system was renewed and the free-coupon program was started), was available at an individual level. Only the screening rates aggregated by age groups of 20–24, 25–29, 30–34, 35–39, and 40–44 years were recorded for the year 2008. In Toyonaka, participants in the regular cervical cancer screening program typically paid ¥600 (about $6 USD) for a standard cervical cancer screening.

The rate of cervical cancer screening among the young generation of women (defined here as women aged 20–44 years) for each year between 2009 and 2012 was analyzed. During the period from 2009 to 2012, a free-coupon program was conducted for women at 5-year age intervals, beginning at the recommended starting age of 20 years (ie, ages 20, 25, 30, 35, and 40 years). These screening rates were compared to that of each age group during the index year of 2008, which was just prior to the start of the free-coupon program. A comparison of the rates for those requiring further diagnostic workups and for cancer detections between the free-coupon and regular screening programs was also conducted. The screening histories of the free-coupon group and regular screening program group were analyzed for changes in consecutive screening rates and any links between those rates and the screening sites where the previous screening was performed.

This study was approved by the Institutional Review Board and the Ethics Committee of the Osaka University Hospital.

Statistical analysis

MedCalc software (MedCalc Software, Mariakerke, Belgium) was used for the statistical analysis. Increases in the screening rate for each age or age group were evaluated by the logistic regression model. Differences in the rates of further diagnostic workups and cancer detection between the free-coupon group and the regular screening group were evaluated using Fisher's exact test. Differences in consecutive screening rates between a free-coupon group and a regular screening group and between screening sites were also evaluated using Fisher's exact test. Results were considered to be significant when the *P*-value was less than 0.05.

RESULTS

Effect of a free-coupon on young women's participation in cervical cancer screening

Figure and Table 1 show the yearly rate of cervical cancer screening for 20- to 44-year-old women between the years of 2009 and 2012, when the free-coupon program was being conducted. The screening rates for free-coupon-eligible 20-, 25-, 30-, 35-, and 40-year-old women formed peaks. Compared to screening rates in the year 2008 (prior to the free-coupon program), which were calculated for the age groups of 20–24, 25–29, 30–34, 35–39, and 40–44 years, the screening rates for the 20-, 25-, 30-, 35-, and 40-year-old women exhibited statistically significant increases (rate ratio [RR] 7.1, 95% confidence interval [CI] 5.9–8.6; RR 6.4, 95% CI 5.2–7.1; RR 3.1, 95% CI 2.9–3.3; RR 3.3, 95% CI 3.1–3.5; and RR 3.0, 95% CI 2.8–3.2, respectively; Table 2). The RRs of the 20- and 25-year-olds were especially high, relative to those of the 30-, 35-, and 40-year-olds.

Effect of a free-coupon program on participation rates in cervical cancer screening by the ineligible population

Interestingly, the screening rates for the coupon-ineligible population also increased during the study period (Figure).

Table 1. Yearly rate of cervical cancer screening for 20- to 44-year-old women between the years of 2008 and 2012

Age (years)	2008	2009	2010	2011	2012
20		174/2016 (8.6%)	183/1868 (9.8%)	220/1731 (12.7%)	175/1778 (9.8%)
21		24/1921 (1.2%)	40/1994 (2.0%)	25/1879 (1.3%)	69/1746 (4.0%)
22	137/9573 (1.4%)	34/1989 (1.7%)	54/1950 (2.8%)	63/2006 (3.1%)	76/1910 (4.0%)
23		51/2077 (2.5%)	76/2015 (3.8%)	60/1960 (3.1%)	101/2004 (5.0%)
24		44/2082 (2.1%)	86/2071 (4.2%)	65/1997 (3.3%)	108/1925 (5.6%)
25		408/2290 (17.8%)	409/2049 (20.0%)	495/2091 (23.7%)	440/2003 (22.0%)
26		78/2240 (3.5%)	89/2237 (4.0%)	79/2068 (3.8%)	133/2104 (6.3%)
27	360/11 031 (3.3%)	85/2293 (3.7%)	154/2255 (6.8%)	136/2241 (6.1%)	168/2110 (8.0%)
28		100/2335 (4.3%)	151/2328 (6.5%)	156/2246 (6.9%)	184/2311 (8.0%)
29		145/2473 (5.9%)	205/2364 (8.7%)	185/2385 (7.8%)	239/2279 (10.5%)
30		578/2628 (22.0%)	639/2494 (25.6%)	616/2393 (25.7%)	593/2518 (23.6%)
31		235/2793 (8.4%)	249/2578 (9.7%)	199/2541 (7.8%)	282/2390 (11.8%)
32	1032/13 232 (7.8%)	170/2836 (6.0%)	247/2765 (8.9%)	220/2627 (8.4%)	226/2602 (8.7%)
33		278/2952 (9.4%)	349/2858 (12.2%)	284/2775 (10.2%)	317/2654 (11.9%)
34		208/3233 (6.4%)	269/3019 (8.9%)	174/1896 (6.0%)	274/2801 (9.8%)
35		874/3574 (24.5%)	873/3219 (27.1%)	863/3054 (28.3%)	736/3016 (24.4%)
36		244/3404 (7.2%)	219/3468 (6.3%)	158/3283 (4.8%)	212/3079 (6.9%)
37	1334/16 753 (8.0%)	381/3558 (10.7%)	389/3460 (11.2%)	362/3415 (10.6%)	375/3308 (11.3%)
38		223/3335 (6.7%)	299/3579 (8.4%)	238/3480 (6.8%)	319/3465 (9.2%)
39		322/3314 (9.7%)	352/3357 (10.5%)	352/3526 (10.0%)	374/3462 (10.8%)
40		807/3422 (23.6%)	832/3309 (25.1%)	865/3361 (25.7%)	773/3599 (21.5%)
41		346/3223 (10.7%)	312/3362 (9.3%)	294/3308 (8.9%)	266/3386 (7.9%)
42	1277/15 900 (8.0%)	196/2607 (7.5%)	240/3234 (7.4%)	239/3379 (7.1%)	242/3293 (7.3%)
43		349/2932 (11.9%)	262/2594 (10.1%)	330/3240 (10.2%)	395/3371 (11.7%)
44		214/3014 (7.1%)	233/2954 (7.9%)	174/2612 (6.7%)	244/3248 (7.5%)

Compared with the screening rate in 2008, the screening rates in the off years from 2009 to 2012 for the coupon-ineligible women in the 21–24, 26–29, 31–34, 36–39, and 41–44 year age groups also significantly increased at the same time that the free-coupon was sent to the eligible 20-, 25-, 30-, 35-, and 40-year-old women (Table 1). The RRs for the 21–24 and 26–29 year age groups were around 2.0 (RR 2.2, 95% CI 1.8–2.6 and RR 1.9, 95% CI 1.7–2.1, respectively); however, those of the 31–34, 36–39, and 41–44 year age groups were around 1.1 (RR 1.2, 95% CI 1.1–1.2; RR 1.1, 95% CI 1.1–1.2; and RR 1.1, 95% CI 1.0–1.2; Table 2).

In order to analyze the reasons for the increased screening rates observed among coupon-ineligible women, the screening history of members of the ineligible population (ie, 21-, 22-, 23-, 24-, 26-, 27-, 28-, and 29-year-old women) post-2009, when the free-coupon program started, who attended screening in 2012 ($n = 799$) was investigated (Table 3). Among 799 women, excluding in-migrants, 531 (66%) had no prior history of screening, while 156 (20%) had a history of an ordinary program screening alone, and 111 (14%) had a history of a free-coupon program screening.

Quality evaluation of cervical cancer screening in a free-coupon program

In order to compare the characteristics of women who received a free-coupon screening and those who were screened in a regular program, the rate of further diagnostic workups and that of cancer detection were analyzed in both groups. The women aged 20, 25, 30, 35, and 40 years were all eligible for a free-coupon, so there were no women among these groups who received a regular program screening and who paid for the costs. The rates of further diagnostic workups and cancer detection during 2009 to 2012 were compared between the women aged 20, 25, 30, 35, and 40 years who received screening with a free-coupon versus those aged 21, 26, 31, 36, and 41 years who received screening in a regular paid program. The rate of requiring further diagnostic workups was 2.0% (240/11 793) in the free-coupon group and 2.3% (80/3553) in the regular program group, indicating no significant difference between the two groups ($P = 0.43$ by Fisher's exact test). The rate of cancer detection was 8.4 per 100 000 (10/11 793) in the free-coupon group and 8.9 per 100 000 (3/3553) in the regular program group, indicating no significant difference between the two groups ($P = 1.0$ by Fisher's exact test).

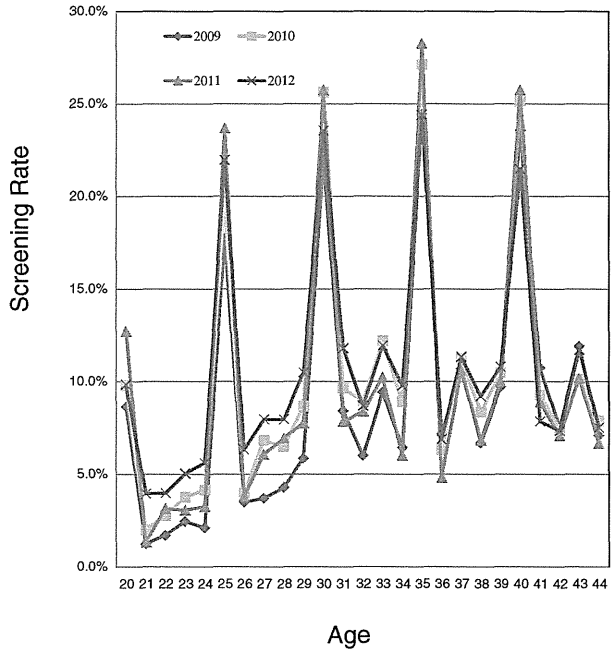

Figure 1. The rate of cervical cancer screening in women 20 to 44 years old in Toyonaka between 2009 and 2012.

Rate of consecutive cervical cancer screening after a free-coupon screening

The screening rates of the women aged 20 and 25 years were dramatically increased by the free-coupon program (Figure and Table 1). To assess whether these increased screening rates resulted in increased rates of consecutive screening, the data were analyzed regarding whether or not those women who underwent a free-coupon screening at the ages of 20 or

Table 2. Comparison of the cervical cancer screening rate between the index year of 2008 and the free-coupon program years of 2009–2012

Age, years	2008	2009–2012		
	Rate of screening	Rate of screening	Rate ratio	95% CI
20	1.4%	10.2%	7.1	5.9–8.6
21–24		3.1%	2.2	1.8–2.6
25	3.3%	20.8%	6.4	5.7–7.1
26–29		6.3%	1.9	1.7–2.2
30	7.8%	24.2%	3.1	2.9–3.3
31–34		9.0%	1.2	1.1–1.2
35	8.0%	26.0%	3.3	3.1–3.5
36–39		8.8%	1.1	1.1–1.2
40	8.0%	23.9%	3.0	2.8–3.2
41–44		8.7%	1.1	1.0–1.2

Table 3. Past screening history of the population ineligible for a free coupon who received a screening in a regular local program in 2012

Age, years	Number screened (in 2012)	Fixed domicile resident	No history of screening	History of screening with free-coupon	History of screening without free coupon
21	69	65	56 (86%)	9 (14%)	0 (0%)
22	76	65	56 (86%)	9 (14%)	0 (0%)
23	101	79	54 (68%)	19 (24%)	6 (8%)
24	108	90	72 (80%)	0 (0%)	18 (20%)
Subtotal	354	299	237 (80%)	37 (12%)	24 (8%)
26	133	112	81 (72%)	5 (4%)	26 (23%)
27	168	90	28 (31%)	40 (44%)	22 (24%)
28	184	134	88 (66%)	29 (22%)	17 (13%)
29	239	164	97 (59%)	0 (0%)	67 (41%)
Subtotal	724	500	294 (59%)	74 (15%)	132 (26%)
Total	1078	799	531 (66%)	111 (14%)	156 (20%)

Table 4. Rates of consecutive cervical cancer screening after a free-coupon screening and a regular screening

	Screening number	Out-migrant within 2 years	Repeated screening within 2 years
Free coupon in 2009			
20 years old	174	19/174 (11%)	10/152[a] (6.5%)[b]
25 years old	408	92/408 (23%)	40/311[a] (13%)[c]
Total	582	111/582 (19%)	50/463[a] (11%)[d]
Regular program in 2009			
21 years old	24	3/24 (13%)	7/21[a] (33%)[b]
26 years old	78	17/78 (22%)	18/61[a] (30%)[c]
Total	102	20/102 (20%)	25/82[a] (30%)[d]

[a]Cases that required further diagnostic workups on initial screening are excluded.
[b,c,d]$P < 0.001$ by Fisher's exact test.

Table 5. Differences in rates of consecutive screening are related to the screening sites where the previous screening was performed

	Clinic A	Other institutions	*P*-value
Free coupon in 2009			
Subsequent screening within 2 years	22/88 (25%)[b]	28/375[a] (7%)[b]	<0.001
Ordinary program in 2009			
Subsequent screening within 2 years	13/32 (41%)[c]	12/50[a] (24%)[c]	0.11

[a]The cases that required further diagnostic workups on initial screening were excluded.
[b]$P < 0.001$ by Fisher's exact test.
[c]$P = 0.07$ by Fisher's exact test.

25 years returned for a subsequent screening. The rate of consecutive cervical cancer screening was compared between the women aged 20 and 25 years who received screening with a free-coupon in the year 2009 and those aged 21 and 26 years who received screening in a regular program in 2009.

In order to investigate the rate of consecutive screening, we excluded from analysis women who out-migrated after a free-coupon screening. In the urban city of Toyonaka, the number of out-migrants was relatively high. Among 582 women aged 20 or 25 years who received a free-coupon screening in the year 2009, 111 persons (19%) moved out of the city within 2 years (Table 4). Among the 102 coupon-ineligible women aged 21 or 26 years who received a screening in a regular program in the year 2009, 20 persons (20%) moved out of the city within 2 years.

After excluding the out-migrants, the continuous screening rate was analyzed. In Japan, women aged 20 years or older are invited for cervical cancer screenings at consecutive two-year intervals, with financial support from their local government. The consecutive screening rate of women aged 20 and 25 within the 2-year interval following the introduction of the free-coupon screening program in 2009 was 6.5% for the 20-year-olds (10/152) and 13% for the 25-year-olds (40/311). On the other hand, the rates of re-visits for women aged 21 or 26 years within a similar 2-year period following a screening in the regular program in the year 2009 were significantly higher: 33% for the 21-year-olds (7/21; $P < 0.001$) and 30% for the 26-year-olds (18/61; $P < 0.001$).

When for some reason a person does not receive a screening after a 2-year interval, she can still undergo a screening in the 3rd year with the same financial support. The consecutive screening rate of women aged 20 and 25 within the 3-year interval following the introduction of the free-coupon screening program in 2009 was 16% for the 20-year-olds (24/142) and 22% for the 25-year-olds (63/277; data not shown). On the other hand, the rates of re-visits for women aged 21 or 26 years within a similar 3-year period following screening in the regular program in the year 2009 were significantly higher: 56% for the 21-year-olds (10/18; $P < 0.001$) and 60% for the 26-year-olds (31/52; $P < 0.001$; data not shown).

Effect of screening site on rate of repeating cervical cancer screening

Next, we investigated the effect of where the screening tests were performed on the consecutive screening rate of women aged 20 or 25 years who received a free-coupon screening and that of those aged 21 or 26 years who received a screening through the regular program in 2009. There were 22 clinics and 6 screening centers where cervical screening test were provided in Toyonaka; however, only 18 of the 22 clinics participated in the 2009 program.

Interestingly, the consecutive screening rates of the 20- and 25-year-olds screened for free at clinic A within the 2-year interval was 25% (22/88), which was significantly higher than the 7% (28/375) reported from the other institutions ($P < 0.001$; Table 5). On the other hand, the consecutive screening rates for 21- and 26-year-olds after a paid screening were slightly (but not significantly) higher at clinic A than at the other screening sites ($P = 0.11$).

The consecutive screening rates of the 20- and 25-year-olds screened for free at clinic A within the 3-year interval was 46% (37/80), which was significantly higher than the 15% (50/339) reported from the other institutions ($P < 0.001$; data not shown). On the other hand, the consecutive screening rates for 21- and 26-year-olds after a paid screening were slightly (but not significantly) higher at clinic A than the other screening sites ($P = 0.07$).

DISCUSSION

There is a critical need to improve the rate of cervical cancer screening among younger women in Japan, as well as in many developing countries. The screening rate of women aged 20 to 29 years is still less than 10%,[4] despite the increasing incidence of cervical cancer in this group.[5] In addition, due to a media blitz about adverse events following HPV vaccination and a statement by the Ministry of Health, Labor, and Welfare of Japan in June 2013 regarding the suspension of an aggressive recommendation for HPV vaccination, the rate of HPV vaccination has dramatically decreased. Given these

situations, the need for improvement in the cervical cancer screening rate among younger women is attracting serious attention. National and local governments therefore enacted a program in which a free cervical screening coupon was sent to 20-, 25-, 30-, 35-, and 40-year-old women to address this problem.

Although many interventions have attempted to remove some of the barriers to cervical cancer screening,[10–16] out-of-pocket costs for screening remain a barrier to access in the United States and Japan.[7] Recently, Tabuchi et al. demonstrated that removal of the out-of-pocket costs by providing a free-screening coupon improved cervical cancer screening participation in Japan.[6] However, they did not analyze how the screening rate was affected for women who had out-of-pocket costs (because of ineligible age for the free screening). In the present study, the screening rates during 2009 to 2012 were shown to rise sharply among those receiving free screening compared to the rates among those of the same age during the pre-program index year of 2008, especially in the two youngest age groups studied (ie, the women aged 20 or 25; Figure and Table 1). However, the screening rate among coupon-eligible women did not increase significantly between 2009 and 2012 (data not shown). This might imply a limitation of the effect of removal of out-of-pocket costs.

We demonstrated for the first time that the screening rates of the population who were paying for their screening (because they were an ineligible age) also increased significantly during the period of this program. While the rates among coupon-ineligible women did not increase as dramatically as those among coupon-eligible women, there was still a significant improvement over 2008 rates.

Possible reasons for the increased screening rates of the youngest of the free-coupon ineligible population during the free-coupon program might be an return visit for screening in a regular program 1 to 3 years after an initial free-coupon screening, or due to indirect effects of the free-coupon program, including improved education and understanding of cervical (and breast) cancer and enhanced motivation for cancer screening. Peer pressure from family, friends, and colleagues to participate in screening between members of the two groups is also likely.

The rate of repeat screening after receiving a previous free-coupon screening among the women who received a regular screening in 2012 was only 14%. This low rate of repeat screening suggests that the significant increase of screening rates seen among 21- to 24-year-old and 26- to 29-year-old women (RR 2.2 and 1.9, respectively; Table 2) cannot be explained by return visits for a regular screening 1 to 3 years after initial free-coupon screening. The increased screening rates of the ineligible population after the free-coupon program started might be caused by indirect publicity effects of the free-coupon program, including improved understanding of cervical cancer and enhanced motivation for cancer screening in young women (Table 2). This somewhat unexpected effect of the free-coupon program should be confirmed in the future.

It was also demonstrated that the rate of requiring a diagnostic workup and the rate of cancer detection due to the screenings were not markedly different between the free-coupon and paid screening program groups. Perhaps more importantly, it was demonstrated for the first time that the follow-up screening rates were significantly lower in the free-coupon group than in the regular screening group (Table 4). This result shows that the complete removal of out-of pocket costs for cervical cancer screening dramatically inspires young women to attend an initial screening; however, it does not translate to following through for a repeat screening 2 years later. This may be a limitation of the effect of a free-coupon cervical cancer screening program. On the other hand, the women who paid some amount of money for a regular screening program were shown to have a consecutive screening than those who attended a free-coupon screening. These results suggest that the largest problem now is how to inspire women to maintain a regular schedule of subsequent screenings. Understanding why the free-coupon group failed to improve rates of consecutive screening will help in providing a solution.

Interestingly, the consecutive screening rate after a free-coupon screening varied depended on where the participants received their previous screening test. This link to the screening experience may provide a partial explanation for the lack of improvement in consecutive screening rates. In the clinic where the rate of follow-up screening was significantly higher, the doctors and staff had spent enormous time and effort to educate the patient about the importance of the screening test to detect cervical cancer; however, it is difficult to statistically compare these educational efforts with those of other institutions. Education is but a part of the screening experience. Institutional reputation, location, scheduling convenience, and waiting room and screening room ambiance all play a role in whether the patient perceives the screening experience as worth repeating. These features of the screening experience are all difficult to quantify and compare statistically.

The Community Preventive Services Task Force demonstrated effectiveness of removal of out-of-pocket costs for breast cancer screening in increasing screening rates for breast cancer; however, evidence with respect to improving cervical cancer screening rates was insufficient.[17] The present study provided some evidence that a free-coupon program is also effective in improving cervical cancer screening rates.

In the present study, the effects of a free-coupon program on the screening rate of both eligible and ineligible women, the rates of requiring further diagnostic workups and cancer detection of a free-coupon screening, and the consecutive screening rate following a free-coupon screening in Toyonaka were analyzed. However, data from only one urban city were analyzed, which is a limitation of the present study. A larger, nation-wide study is necessary to confirm our findings.

ONLINE ONLY MATERIAL

Abstract in Japanese.

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RESEARCH ARTICLE

Molecular Characterization of an Intact p53 Pathway Subtype in High-Grade Serous Ovarian Cancer

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Data Availability: The authors confirm that, for approved reasons, some access restrictions apply to the data underlying the findings. Data contain identifying information and cannot be made available. A de-identified minimal dataset is available at Figshare: http://dx.doi.org/10.6084/m9.figshare.1235612. Additional data are available upon request to Prof. Ituro Inoue (itinoue@nig.ac.jp)

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Abstract

High-grade serous ovarian cancer (HGSOC) is the most aggressive histological type of epithelial ovarian cancer, which is characterized by a high frequency of somatic *TP53* mutations. We performed exome analyses of tumors and matched normal tissues of 34 Japanese patients with HGSOC and observed a substantial number of patients without *TP53* mutation (24%, 8/34). Combined with the results of copy number variation analyses, we subdivided the 34 patients with HGSOC into subtypes designated ST1 and ST2. ST1 showed intact p53 pathway and was characterized by fewer somatic mutations and copy number alterations. In contrast, the p53 pathway was impaired in ST2, which is characterized by abundant somatic mutations and copy number alterations. Gene expression profiles combined with analyses using the Gene Ontology resource indicate the involvement of specific biological processes (mitosis and DNA helicase) that are relevant to genomic stability and cancer etiology. In particular we demonstrate the presence of a novel subtype of patients with HGSOC that is characterized by an intact p53 pathway, with limited genomic alterations and specific gene expression profiles.

Introduction

The age adjusted rates of ovarian and other uterine adnexa cancers in 2002 were 10.6 per 100,000, and 5.2 per 100,000 person-years in USA and Japan, respectively [1]. Epithelial ovarian cancer is a heterogenous entity comprising multiple

histological types such as high-grade serous, low-grade serous, clear cell, endometrioid, and mucinous cancers [2, 3]. Ovarian cancers are divided into Type I and Type II tumors [2, 4]; Type I tumors include low-grade serous, low-grade endometrioid, clear-cell, and mucinous carcinomas. These tumors poorly respond to platinum-based therapy, harbor a high frequency of mutations in genes that encode components of the RAS signaling pathway, and are relatively stable in genomic structure. Type II tumors include high-grade serous and high-grade endometrioid carcinomas and are highly aggressive. A large-scale study of high-grade serous ovarian cancer (HGSOC) by The Cancer Genome Atlas (TCGA) group characterized HGSOC as *TP53*-mutation enriched (96%) with aberrations of genome-wide somatic gene copy numbers. This study identified commonly altered pathways such as RB1, PI3K/RAS, NOTCH, homologous recombination, and FOXM1 pathways [5]. The mutation status of *TP53* is associated with stages, gene expression patterns, and the survival of patients with serous ovarian cancer [6].

We attempted to establish a risk classification system for serous ovarian cancer using gene expression profiles acquired using microarray data [7, 8]. We identified 88 genes related to progression-free survival in 110 Japanese patients with advanced-stage serous ovarian cancer [7], as well as 126 genes related to overall survival in 260 Japanese patients with advanced-stage HGSOC [8]. To provide a better understanding of the molecular mechanisms involved in the pathogenesis of these cancers and to develop a risk classification system, we conducted profiling of the somatic mutations present in these tumors.

We compiled genomic information for patients with HGSOC using exome sequencing and copy number variation (CNV) analyses. According to the profiles of somatic single nucleotide variants (SNVs), small insertions and deletions (indels), and CNVs, we classified HGSOC into subtypes designated ST1 and ST2 that are characterized by intact or impaired p53 signaling pathways, respectively. We further characterized the two subtypes by comparing their gene expression profiles. Gene ontology (GO) analysis showed that differentially expressed genes were significantly enriched in the mitosis and DNA helicase GO groups that may be involved in genomic instability and tumorigenesis of HGSOC. These findings provide new insights into the molecular characteristics and novel biological processes that contribute to the pathogenesis of HGSOC, particularly in patients with an intact p53 pathway.

Materials and Methods

Ethics statement

The ethics committees of Niigata University (IRB No. 239, 428, and 455) and National Institute of Genetics (IRB No. 23-11) approved the study protocols, and each participant provided written informed consent for the collection of samples and subsequent analyses.

Clinical samples

Fresh-frozen samples were obtained from primary tumor tissues before administration of chemotherapy. Two pathologists assessed the histological characteristics of formalin-fixed and paraffin-embedded hematoxylin and eosin sections. Because definite histological characterization was a critical component of the study, a central pathological review was conducted by two independent gynecologic pathologists (HT and TM) with no knowledge of the patients' clinical status. Histological types and degree of histological differentiation were determined according to the WHO classification of ovarian tumors and Silverberg classification, respectively [8]. Clinical data (pT- and FIGO-stage) are shown in Table S1. We used peripheral blood as the matched normal tissue.

Exome sequencing

Genomic DNA was isolated from tumor tissues using a phenol-chloroform method and from peripheral blood using the QIAamp DNA Blood Maxi Kit (QIAGEN) [8]. Genomic DNA was hybridized with SureSelect Human All Exon Kits (Agilent) to prepare sequencing libraries, and the libraries were sequenced using the Illumina HiSeq 2000 (Illumina) with 90 or 100 base-paired end modules. Sequence reads were aligned to a reference genome (UCSC hg19) using BWA [9] and SAMtools [10]. Picard (http://picard.sourceforge.net) was used for removing duplicate reads. Local realignment of reads around known indels and recalibration of base quality were performed using GATK [11]. The heuristic somatic mutation caller, VarScan 2 [12], was used for somatic mutation calling. Threshold criteria for detecting somatic mutations were as follows: normal variant frequency of 0% and Fisher's exact test p value of <0.00001. Functional information of somatic mutations was annotated using ANNOVAR [13] and Oncotator (http://www.broadinstitute.org/oncotator/).

Prediction of functional impacts of missense single nucleotide variants

Functional effects of the identified somatic missense mutations were evaluated using MutationAssessor 2 [14], which predicts the effect of amino acid substitutions according to a pattern of evolutionary conservation based on multiple sequence alignments of a protein family. Missense mutations with a functional impact score (FIS) of >2.0 were defined as deleterious.

Detection of cancer driver genes

To detect possible cancer driver genes based on the identified somatic mutations, we used OncodriveFM [15], which evaluates the accumulation of mutations with high functional impact within a gene, assuming that cancer driver genes are highly mutated and exert substantial functional impacts. However, the consequences of

mutations in passenger genes are mostly benign. OncodriveFM derives FIS from the MutationAssessor 2 to assess whether mutated genes are drivers or passengers.

Analyses of CNV and tumor purity

Single nucleotide polymorphism (SNP) array experiments using Genome-Wide Human SNP 6.0 (Affymetrix) were previously conducted for 30 of 34 HGSOC samples [8, 16]. Because of the technical difficulties and limited DNA amounts, we could not obtain SNP array data for remaining four samples. Affymetrix CEL files from SNP array experiments using 30 samples were processed using the CNV detection software package PennCNV [17]. CNVs were called using a hidden Markov model according to calculations of the log R ratio and B-allele frequency values. The CNV frequency between tumor and normal samples was evaluated for each SNP using Fisher's exact test in the ParseCNV algorithm [18]. Threshold criteria for recurring CNV regions (CNVRs) were as follows: Fisher's exact test p value of <0.0005 and no overlap with structural variations in samples from healthy subjects [19]. In addition, the CEL files were used to estimate tumor purity. We used the ASCAT (Allele-Specific Copy number Analysis of Tumors) algorithm [20] in the NEXUS copy number software version 6.0 (BioDiscovery) [21] to estimate the extent of contaminations of normal cells in tumor samples. The MIAME-compliant SNP array data were deposited to the Gene Expression Omnibus data repository (accession number GSE61237).

Microarray experiments and data processing

Extraction of RNA, Cy3 labeling, microarray hybridization, signal scanning, and feature extraction were performed in previous studies [7, 8]. Data normalization was performed using the GeneSpringGX11 (Agilent) setting of raw signal threshold of 1.0 and normalization to the 75th percentile.

Gene expression analysis

The significance of differences in gene expression between the two subtypes was evaluated using the *t*-test. After the evaluation, multiple testing was corrected by the false discovery rate (FDR) using the Benjamini-Hochberg procedure [FDR (BH)]. We set FDR (BH) to <0.1 as the significant threshold. These analyses were performed using the ComparativeMarkerSelection module of GenePattern [22].

GO analysis

GO analysis was performed using the Functional Annotation Clustering tool included in the DAVID bioinformatics resource [23]. This tool assesses the similarity of annotation terms using kappa statistics and forces groups to share similar annotation profiles using a fuzzy heuristic multiple-linkage partition [24]. Settings were as follows: eight annotation categories (OMIM_Disease, COG_Ontology, SP_PIR_Keywords, GOterm_BP_FAT, GOterm_MF_FAT,

BBID, BioCarta, and KEGG_Pathway), similarity term overlap of ≥ 3, kappa statistic threshold of 1, group membership of ≥ 3, and the fuzzy multiple-linkage partition threshold of 1, respectively. Enrichment scores were calculated using the geometric mean of the modified Fisher's exact test p values ($-$log scale) for gene enrichment of each GO term in each GO group and an enrichment score of >1.3 is considered significant [23].

Data visualization

Somatic mutation data were displayed using Gitools (version 1.8.4) [25]. Copy number data were displayed using Integrative Genomics Viewer (IGV, version 2.3.25) [26]. Bee swarm and box plots were created using the beeswarm package in the CRAN repository (http://cran.r-project.org/). Heat map views of gene expression data were displayed using HeatMapViewer module in GenePattern [22].

Results

Genomic alteration profiling

The somatic mutations identified in samples acquired from 34 Japanese patients with HGSOC were catalogued according to the analysis of exome sequencing data. The average read depth was $91\times$ and $84\times$ for tumor and normal samples, respectively. Coverage of $\geq 10\times$ was achieved for 89% and 88% of coding bases of tumor and normal samples, respectively (Table S2). We identified 1,399 somatic nonsynonymous (missense and nonsense) and splice site mutations (41 mutations per sample) using VarScan 2 [12] with the predefined criteria described in the Materials and Methods section. Of these somatic variants, 158 were randomly selected and subjected to Sanger sequencing, and 143 variants were successfully validated (143/158, 91%). All *TP53* somatic nonsynonymous and splice site mutations were called and validated using VarScan 2 and Sanger sequencing, respectively. For nine patients with no *TP53* somatic nonsynonymous and splice site mutations, we further performed Sanger sequencing for all of the ten *TP53* coding exons because false negative might be expected due to existing low depth reads. We detected a frame-shift deletion on exon 3 for S022 (Table S3). Somatic SNVs and indels were annotated to 1,405 in 1,159 genes. *TP53* was the most frequently mutated (76%, 26/34) (Figure 1A), however the mutation frequency was lower than previous reports [5, 27]. There were 24 distinct and diverse *TP53* mutations (Table S4). Two patients (S066 and S271) shared the same missense variant (R273H) and the other two patients (S009 and S017) shared the same nonsense variant (R196*). Of the remaining 22 *TP53* variants, five were frame-shift deletions (A86fs for S020, P27fs for S022, F113fs for S119, S241fs for S006, and E286fs for S118), one was a nonsense variant (Q52* for S015), two were splice site variants (Y126splice for S188 and S261splice for S008), and the remaining 14 were missense (Table S4). FIS for the 15 *TP53* missense variants was

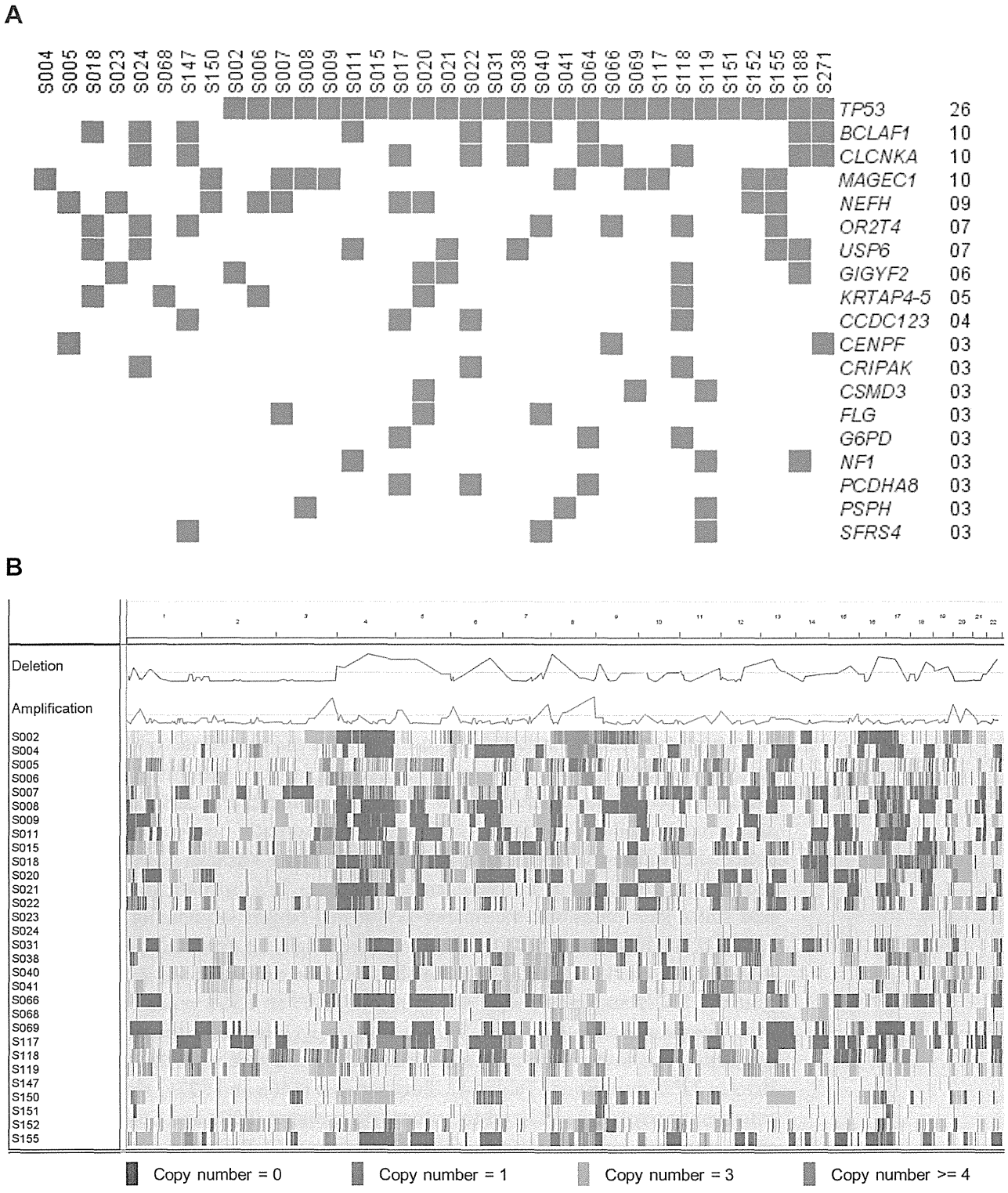

Figure 1. The landscape of genomic alterations in patients with HGSOC. (A) Somatic mutational landscape of 34 patients with HGSOC. Somatic mutations identified in more than or equal to three patients are displayed. Patients with mutations of the same gene are shown in red. (B) Copy number alteration landscape of 30 patients with HGSOC. Copy number (CN) alterations are indicated as follows: CN=0, dark blue; CN=1, light blue; CN=3, pink;

and CN ≥4, red). Blue line in the Deletion track and red line in the Amplification track show copy number alteration frequency. Gray lines in the Deletion and Amplification tracks show the −log-transformed Fisher's exact test p values of 0.0005.

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>2.0 according to MutationAssessor 2 [14] analysis and were designated as deleterious (Table S4).

The second most frequently mutated genes were *BCLAF1*, *CLCNKA*, and *MAGEC1* (29%, 10/34 for each gene). According to FIS determined using MutationAssessor 2, all mutations except K911fs of *BCLAF1* were assessed as benign and were considered passenger mutations. Ninety-two percent (1,063/1,159) of the genes were mutated in one patient. To further explore candidate cancer driver genes mutated in at least two patients, OncodriveFM was applied as described in Materials and Methods section. Only *TP53* was detected as a cancer driver gene with high accumulation of deleterious mutations in our HGSOC samples (data not shown).

CNV profiling for 30 of the 34 HGSOC samples is shown in Figure 1B and File S1. The genome-wide copy numbers of 30 HGSOC samples were altered. ParseCNV identified nine repeatedly deleted CNVRs (1p36.11, 4q24, 5q13.1, 5q13.2, 6q22.33-23.1, 15q24.2-24.3, 17q12, 18q21.31, and 22q12.3) and four amplified CNVRs (1p34.1-33, 3q27.2, 6p24.2, and 10p12.31-12.2) with identified genes, respectively (Tables S5 and S6).

Exclusion of p53 pathway-impaired patients from nonmutated TP53 HGSOC

The *TP53* mutation frequency was significantly lower in our samples compared with those reported in previous studies as follows: 26/34 vs. 301/316; Fisher's exact test p value of 0.0060 [5] and 26/34 vs. 118/126; Fisher's exact test p value of 0.0069 [27]. Among the eight samples with nonmutated *TP53* (Figure 2A), CNV analysis showed heterozygous copy number loss of *TP53* for sample S004 (Figure 2B). MDM2 is an E3 ubiquitin protein ligase that targets p53 for proteasomal degradation and is considered a negative effector of p53 [28]. There is an association between amplification of *MDM2* and loss of p53 function in certain tumors [27]. For the eight samples with intact *TP53*, no *MDM2* copy number amplification was observed (Figure 2A). To further investigate whether an alternative mechanism accounts for p53 dysfunction, we evaluated a list of direct p53 target genes (Table S7) obtained from the Pathway Interaction Database (PID) [29]. We identified an *IRF5* (Interferon Regulatory Factor 5) splice site mutation (W181splice) of sample S018. Overall, we identified six p53 pathway intact patients from the eight patients with HGSOC with nonmutated *TP53* (Figure 2A). We assigned six patients to ST1 and the remaining to ST2.

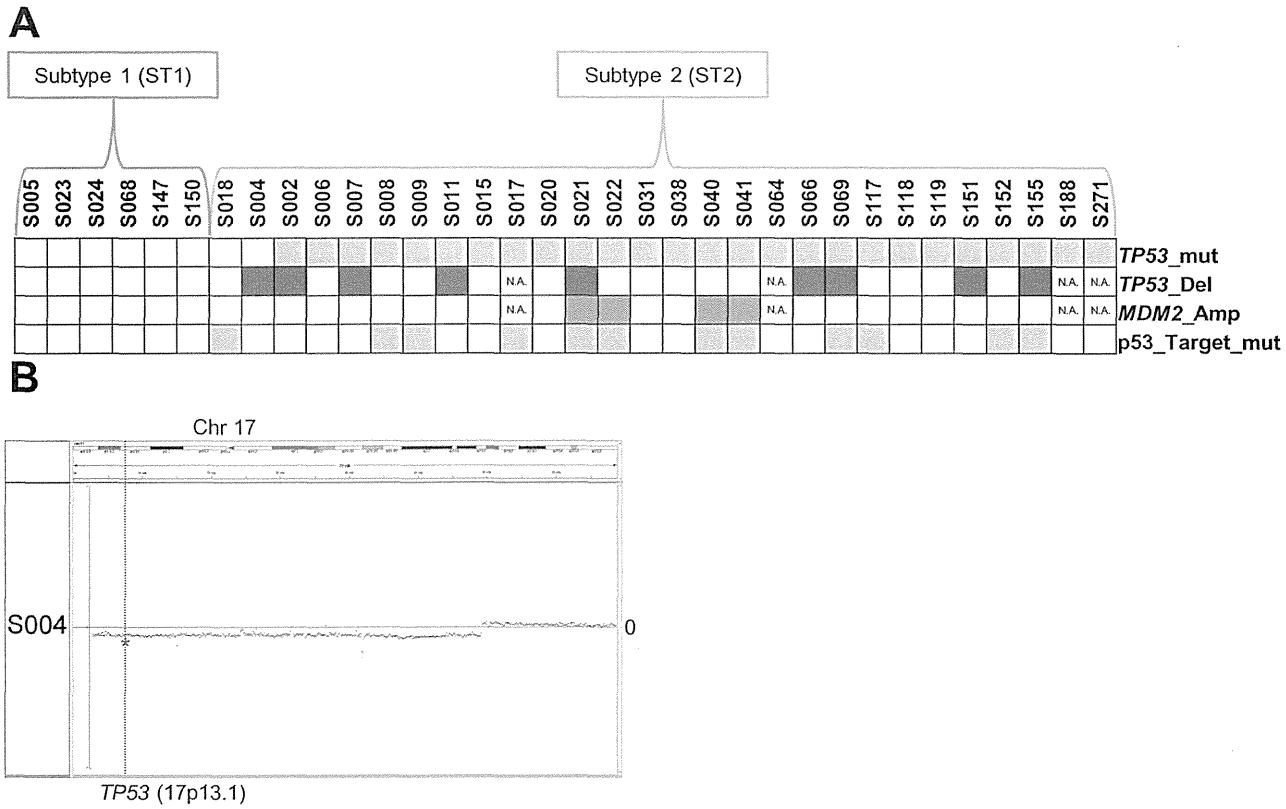

Figure 2. Summary of mutations for *TP53* and p53 pathway genes. (A) Summary of patients with *TP53* mutations are shown in pink in the *TP53*_mut track. *TP53* heterozygous copy number deletions are shown in blue in *TP53*_Del track. *MDM2* copy number amplification is shown in red in the *MDM2*_amp track. Mutations in genes that are direct targets of p53 are shown in green in the p53_Target_mut track. (B) Dot plot of log R ratio (LRR) of Chr17 for sample S004. Blue dots indicate LRR values. The position of line of LRR=0 is indicated as 0 on the right of each graph. *TP53* (17p13.1) is indicated by the blue asterisk on the vertical line.

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Genomic alterations in ST1 and ST2

We did not detect mutations in genes specific for low-grade serous type, such as *BRAF*, *CTNNB1*, *KRAS*, and *PIK3CA* [27], among the 1,159 genes mutated in the 34 HGSOC samples (data not shown).

To characterize differences in genomic alterations between ST1 and ST2, we compared the numbers of somatic nonsynonymous and splice site mutations and found the number of somatic ST1 mutations was significantly lower compared with ST2 (Wilcoxon rank sum test p value of 0.00070) (Figure 3A).

In addition, we compared ST1 and ST2 with respect to the numbers of CNV segments identified by PennCNV [17] in each autosomal chromosome (Table S8). The results of the Wilcoxon rank sum test and multiple test correction for 22 autosomal chromosomes according to false discovery rate (FDR) [30] showed significantly fewer CNV segments on chromosomes 17 and 12 (FDR q value of 0.040 and 0.047, respectively) for ST1 (Figure 3B). These results indicate that ST1

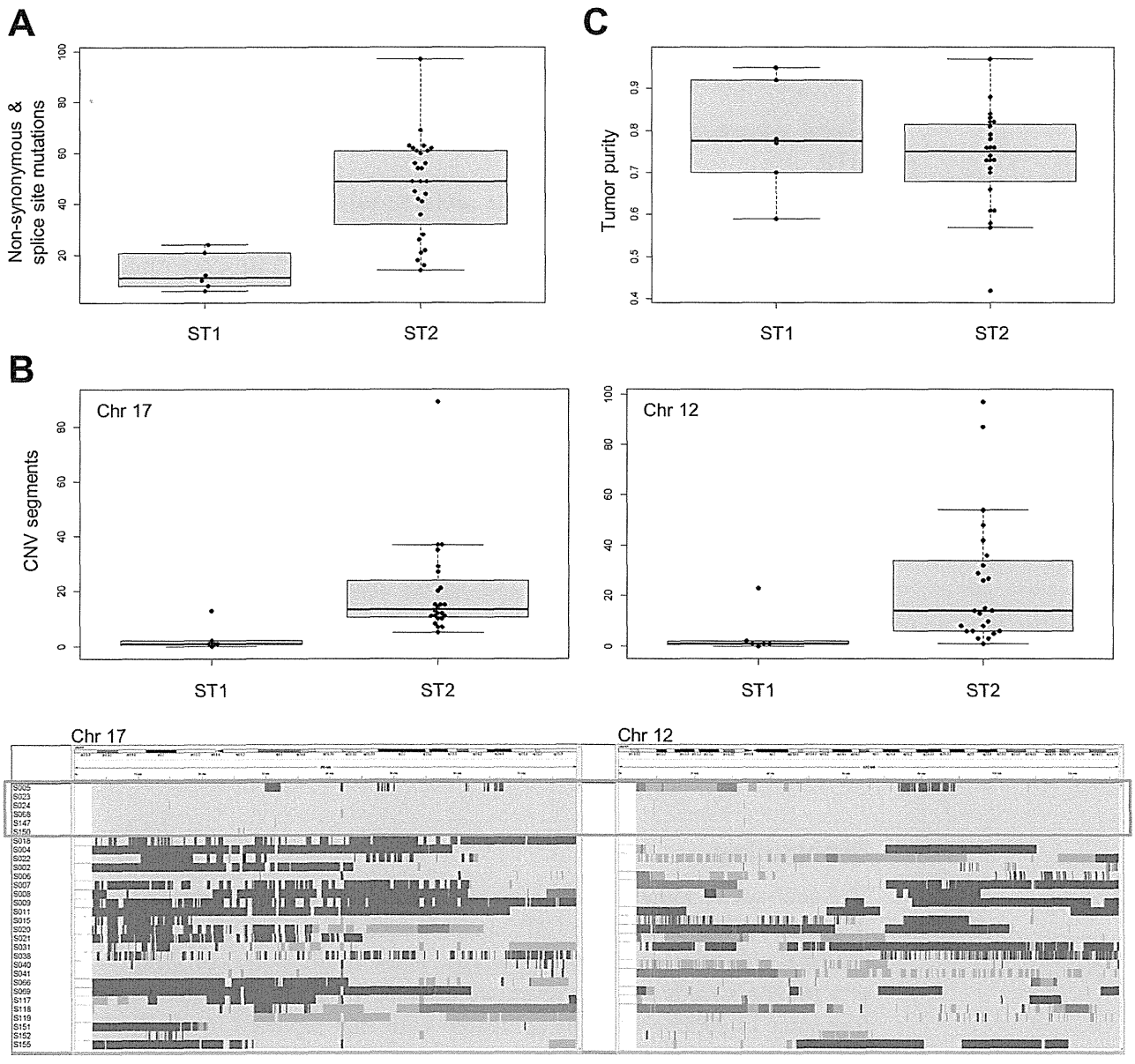

Figure 3. Analysis of genomic alterations in ST1 and ST2. (A) Comparison of the number of somatic nonsynonymous and splice site mutations. (B) Comparison of the number of CNV segments (upper panel) and CNV profiles (bottom panel) on chromosomes 17 and 12 between ST1 and ST2. Copy number alterations are as follows: CN=0, dark blue; CN=1, light blue; CN=3, pink; and CN ≥4, red). ST1 is enclosed by the green rectangle. (C) Tumor purities of ST1 and ST2.

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maintained the normal karyotype and ST2 harbored genome-wide copy number alterations particularly enriched in chromosomes 17 and 12 (Figure 3B).

To exclude the possibility that the low number of mutations and few CNV segments of ST1 were because of a high degree of contamination with normal cells, tumor purity was evaluated as described in Materials and Methods section. The average tumor purities were 79% and 73% for ST1 and ST2, respectively, and

there was no significant difference in tumor purity between subtypes (Wilcoxon rank sum test p value of 0.48) (Figure 3C and Table S9).

Gene expression analysis to functionally characterize ST1 and ST2

The gene expression profiles of ST1 and ST2 were determined using an mRNA microarray [7, 8]. Eighty-nine probes representing 70 genes revealed differences in expression levels between ST1 and ST2 at an FDR (BH) of <0.1 (Tables S10 and S11). The expression levels of 33 and 37 genes were higher (Table S10) and lower (Table S11), respectively, for ST1 compared with that for ST2. The 70 genes showed relatively homogenous and heterogenous expressions in ST1 and ST2, respectively (Figure 4).

To evaluate the biological and functional consequences of the expression of these 70 genes, GO analysis was applied using DAVID. Thirty-five genes were classified into 18 GO groups sharing similar GO terms (Table S12). Two of the 18 GO groups (mitosis and DNA helicase) showed significant enrichment of genes (Enrichment score of >1.3) (Figure 5 and Table S12). *NEK1* and *NEK9* in the mitosis group were upregulated and *ASPM*, *BIRC5*, *CDCA2*, and *SKA3* were downregulated in ST1 compared with that in ST2. *BLM*, *PIF1*, and *RECQL4*, which encode DNA helicases, were expressed at relatively low levels in ST1. Differences in expression of these mitosis and DNA helicase genes were evaluated using the Kolmogorov-Smirnov test, F test, and *t*-test with R version 3.0.2 (Figure 6 and Table S13).

Discussion

The analyses of somatic mutations of HGSOC showed enrichment of *TP53* mutations (Figure 1A). The CNV analysis revealed an altered profile of the genome-wide copy number (Figure 1B). These findings are consistent with those of a previous study [5]. However, we detected a significant difference in the frequency of *TP53* mutations compared with that reported in previous reports [5, 27]. Specifically, eight HGSOC samples did not harbor *TP53* mutations, and mutation of a p53 target gene *IRF5* was identified in one sample. Further, one had *TP53* copy number deletion. Taken together, we assigned six HGSOC samples as ST1 and the remaining 28 samples as ST2.

All of the patients with HGSOC in this study were Japanese while the patients in the previous studies [5, 27] were mainly come from European-descendent populations. The discrepancy of *TP53* mutation frequencies may come from population differences as observed in the case of epidermal growth factor receptor (*EGFR*) mutations for non-small-cell lung cancers [31, 32]. *EGFR* mutation rates were as follows: 11% and 32% in West-European and East-Asian patients, respectively [31], and 2% and 26% of patients in USA and Japan, respectively [32]. The low numbers of patients in the current study compared to the TCGA

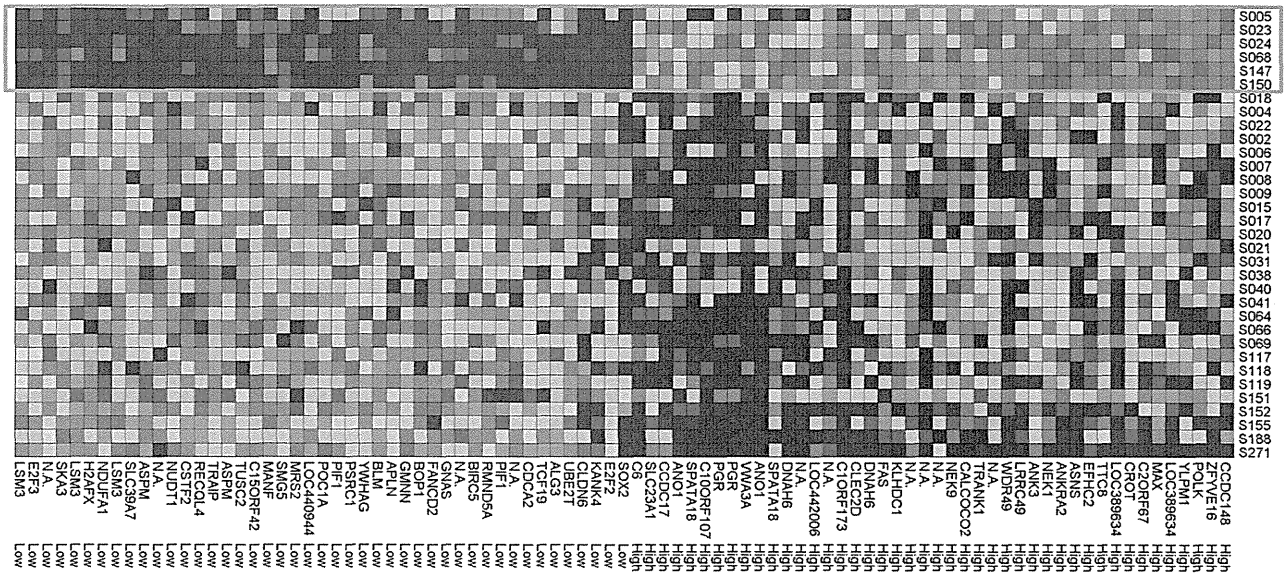

Figure 4. Analysis of gene expression. Seventy genes (89 probes) showing differences at FDR (BH) of <0.1 are displayed. ST1 is enclosed by the green rectangle. High and Low indicate expression levels of ST1 compared with ST2.

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data set [5] may not enough to provide solid conclusion of the *TP53* mutation frequency. Evidently, much larger scale study including Japanese and other Asian patients with HGSOC are needed. The other possibility is the existence of small fraction of *TP53* mutated tumor cells because of tumor heterogeneity in the *TP53* nonmutated patients. It is widely accepted that somatic driver mutations such as mutations of *TP53* occur at an early event of cancer then relatively high frequency of the mutation should be observed. In the current study, we indeed observed at least 20% of tumor variant frequencies for *TP53*. Therefore, we presumably did not overlook driver mutations of *TP53* by the exome sequencing (Figure 1).

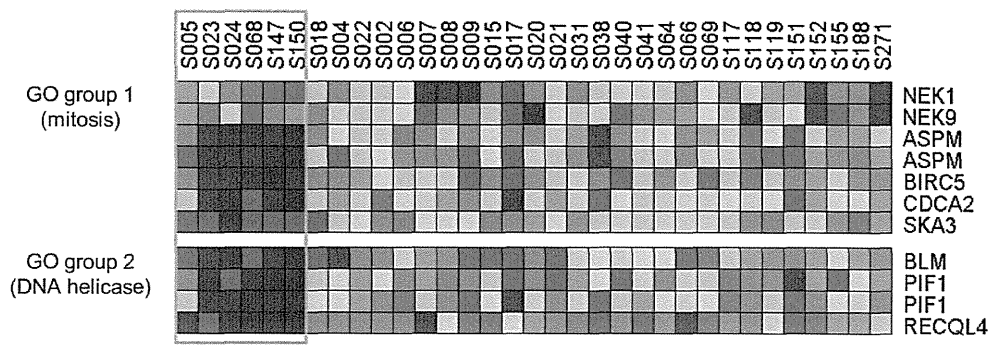

Figure 5. Gene ontology analysis of differentially expressed genes. Heat-map view of gene ontology (GO) groups. Two GO groups (mitosis and DNA helicase) with significant gene enrichment are indicated as GO groups 1 and 2, respectively. ST1 is enclosed by the green rectangle.

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