Table 2 Odds ratio with 95% confidence intervals for individual SNPs in all subjects and in subjects stratified by menopausal status

		All women (n = 936)					Premenopausal (n = 385)					Postmenopausal (n = 551)				
SNP		No. of	Adjusted OR[b]		Multivariate OR[c]		No. of	Adjusted OR[b]		Multivariate OR[c]		No. of	Adjusted OR[b]		Multivariate OR[c]	
Gene/location	Genotype[a]	Case/Control	OR (95% CI)		OR (95% CI)		Case/Control	OR (95% CI)		OR (95% CI)		Case/Control	OR (95% CI)		OR (95% CI)	
rs1562430	CC	7/4	Ref.		Ref.		2/3	Ref.		Ref.			Ref.		Ref.	
/8q24	TC	96/102	0.54	(0.14-1.85)	0.62	(0.15-2.32)	33/42	1.24	(0.19-9.85)	1.10	(0.15-10.05)	5/1	0.24	(0.01-1.54)	0.35	(0.02-2.80)
	TT	369/351	0.61	(0.16-2.05)	0.67	(0.16-2.45)	155/146	1.64	(0.27-12.63)	1.72	(0.24-15.14)	63/60	0.24	(0.01-1.52)	0.29	(0.01-2.25)
	Per allele		1.05	(0.79-1.39)	1.02	(0.75-1.39)		1.08	(0.81-1.45)	1.62	(1.08-2.44)	214/205	1.07	(0.85-1.36)	0.80	(0.56-1.14)
rs889132	AA	76/91	Ref.		Ref.		34/36	Ref.		Ref.			Ref.		Ref.	
MAP3K1/5q	CA	227/211	1.27	(0.89-1.83)	1.27	(0.86-1.88)	91/95	0.96	(0.55-1.65)	0.82	(0.45-1.50)	42/55	1.59	(0.98-2.58)	1.57	(0.91-2.76)
	CC	164/160	1.21	(0.83-1.76)	1.21	(0.81-1.81)	64/61	1.07	(0.60-1.92)	0.98	(0.52-1.84)	136/116	1.35	(0.82-2.23)	1.30	(0.74-2.30)
	Per allele		1.07	(0.89-1.29)	1.07	(0.88-1.31)		1.08	(0.81-1.45)	1.11	(0.83-1.49)	100/99	1.07	(0.85-1.36)	1.05	(0.81-1.36)
rs13283615	AA	75/75	Ref.		Ref.		29/31	Ref.		Ref.			ref.		ref.	
/8q24	GA	211/206	1.04	(0.71-1.51)	1.09	(0.73-1.65)	73/80	0.97	(0.53-1.76)	1.13	(0.60-2.17)	46/44	1.10	(0.68-1.79)	1.17	(0.67-2.05)
	GG	180/177	1.03	(0.70-1.51)	1.02	(0.67-1.55)	86/78	1.14	(0.63-2.05)	1.18	(0.62-2.24)	138/126	0.97	(0.58-1.61)	1.09	(0.61-1.97)
	Per allele		1.01	(0.84-1.21)	1.00	(0.81-1.22)		1.11	(0.84-1.47)	1.03	(1.00-1.05)	94/99	0.95	(0.74-1.21)	0.99	(0.76-1.28)
rs981782	TT	166/149	Ref.		Ref.		67/64	Ref.		Ref.			Ref.		Ref.	
HCN1/5p12	TG	220/234	0.85	(0.64-1.14)	0.82	(0.60-1.13)	88/98	0.85	(0.54-1.33)	0.78	(0.48-1.26)	99/85	0.87	(0.59-1.27)	0.83	(0.54-1.29)
	GG	82/76	0.96	(0.66-1.41)	0.88	(0.58-1.34)	31/28	1.03	(0.56-1.91)	0.97	(0.50-1.90)	132/136	0.93	(0.57-1.52)	0.76	(0.43-1.34)
	Per allele		0.95	(0.79-1.14)	0.97	(0.80-1.17)		1.00	(0.75-1.35)	1.01	(0.74-1.38)	51/48	0.93	(0.73-1.18)	0.86	(0.66-1.13)
rs3803662	CC	74/91	Ref.		Ref.		24/42	Ref.		Ref.			Ref.		Ref.	
TNRC9/16q12	TC	230/227	1.25	(0.88-1.79)	1.32	(0.89-1.96)	89/96	1.59	(0.90-2.85)	1.50	(0.81-2.80)	50/49	1.08	(0.68-1.72)	1.25	(0.73-2.16)
	TT	160/142	1.41	(0.97-2.08)	**1.61**	**(1.06-2.45)**	72/53	**2.29**	**(1.25-4.26)**	**2.29**	**(1.20-4.46)**	141/131	1.04	(0.63-1.71)	1.27	(0.72-2.24)
	Per allele		1.18	(0.98-1.42)	**1.28**	**(1.07-1.55)**		**1.54**	**(1.15-2.09)**	**1.58**	**(1.17-2.16)**	88/89	1.00	(0.78-1.28)	1.07	(0.83-1.39)
rs381798	TT	339/347	Ref.		Ref.		138/140	Ref.		Ref.			Ref.		Ref.	
LSP1/11p15.5	CT	120/107	1.14	(0.85-1.55)	1.07	(0.77-1.49)	46/49	0.92	(0.58-1.48)	1.00	(0.60-1.68)	201/207	1.30	(0.87-1.94)	1.18	(0.75-1.86)
	CC	10/5	2.04	(0.72-6.60)	1.63	(0.52-5.66)	4/1	3.98	(0.58-78.39)	3.29	(0.42-68.89)	74/58	1.65	(0.46-6.55)	1.39	(0.32-6.31)
	Per allele		1.19	(0.91-1.56)	1.11	(0.83-1.49)		1.07	(0.70-1.64)	1.21	(0.77-1.90)	6/4	1.27	(0.90-1.81)	1.14	(0.78-1.66)
rs2046210	GG	213/244	Ref.		Ref.		83/107	Ref.		Ref.			Ref.		Ref.	
ESR1/6q25.1	AG	194/185	1.21	(0.92-1.59)	1.22	(0.90-1.64)	78/72	1.41	(0.92-2.17)	1.63	(1.03-2.61)	130/137	1.11	(0.78-1.59)	0.99	(0.67-1.48)
	AA	61/34	**2.03**	**(1.29-3.25)**	**2.16**	**(1.32-3.59)**	27/14	**2.46**	**(1.23-5.10)**	**2.93**	**(1.40-6.40)**	116/113	1.69	(0.93-3.14)	1.69	(0.84-3.50)
	Per allele		**1.34**	**(1.10-1.63)**	**1.37**	**(1.11-1.70)**		**1.49**	**(1.10-2.03)**	**1.70**	**(1.24-2.35)**	34/20	1.23	(0.95-1.59)	1.14	(0.86-1.51)

Table 2 Odds ratio with 95% confidence intervals for individual SNPs in all subjects and in subjects stratified by menopausal status *(Continued)*

rs909116	CC	166/178	Ref.		Ref.		71/64	Ref.		Ref.			Ref.		Ref.	
LSP/11p15.5	CT	225/228	1.08	(0.81-1.43)	1.04	(0.77-1.42)	88/106	0.76	(0.49-1.18)	0.90	(0.55-1.47)	95/114	1.36	(0.94-1.97)	1.20	(0.79-1.83)
	TT	79/57	1.49	(0.99-2.24)	1.40	(0.90-2.19)	30/23	1.21	(0.64-2.30)	1.23	(0.62-2.48)	137/122	**1.72**	**(1.02-2.90)**	1.69	(0.94-3.09)
	Per allele		1.18	(0.97-1.42)	1.15	(0.93-1.41)		0.98	(0.72-1.32)	1.11	(0.81-1.52)	49/34	**1.32**	**(1.03-1.69)**	1.24	(0.95-1.63)
rs30099	CC	225/216	Ref.		Ref.		93/84	Ref.		Ref.			Ref.		Ref.	
/5q	TC	205/198	0.82	(0.52-1.29)	1.08	(0.80-1.45)	82/84	0.87	(0.57-1.33)	0.96	(0.61-1.53)	132/132	1.08	(0.76-1.54)	1.21	(0.80-1.83)
	TT	42/50	0.99	(0.76-1.30)	0.86	(0.52-1.41)	15/25	0.53	(0.26-1.06)	0.51	(0.24-1.08)	123/114	1.12	(0.61-2.06)	1.19	(0.58-2.45)
	Per allele		0.93	(0.76-1.13)	0.98	(0.79-1.22)		0.78	(0.57-1.06)	0.85	(0.92-1.16)	27/25	1.04	(0.81-1.36)	1.12	(0.83-1.50)
rs2981282	CC	220/226	Ref.		Ref.		86/94	Ref.		Ref.			Ref.		Ref.	
FGFR2 /10q26	TC	210/190	1.15	(0.87-1.50)	1.19	(0.89-1.60)	91/81	1.23	(0.81-1.87)	1.48	(0.94-2.35)	134/132	1.10	(0.77-1.58)	1.08	(0.72-1.62)
	TT	41/45	0.92	(0.58-1.47)	0.84	(0.50-1.40)	13/17	0.89	(0.41-1.92)	1.07	(0.46-2.50)	119/109	0.95	(0.53-1.71)	0.76	(0.38-1.48)
	Per allele		1.03	(0.84-1.25)	1.02	(0.82-1.27)		1.04	(0.75-1.43)	1.27	(0.91-1.78)	28/28	1.04	(0.80-1.34)	0.94	(0.71-1.24)
rs795399	TT	255/249	Ref.		Ref.		90/107	Ref.		Ref.			Ref.		Ref.	
IGF1/12q23.2	CT	180/173	0.84	(0.51-1.36)	1.05	(0.78-1.41)	82/65	1.49	(0.97-2.30)	1.56	(0.98-2.48)	165/142	0.80	(0.56-1.15)	0.78	(0.52-1.18)
	CC	34/41	1.03	(0.78-1.35)	0.85	(0.49-1.45)	15/20	0.86	(0.41-1.77)	1.04	(0.46-2.27)	98/108	0.87	(0.44-1.70)	0.93	(0.43-1.99)
	Per allele		0.96	(0.79-1.18)	0.97	(0.78-1.21)		1.13	(0.83-1.55)	1.25	(0.91-1.72)	19/21	0.87	(0.66-1.14)	0.88	(0.66-1.17)
rs3757318	GG	249/281	Ref.		Ref.		95/111	Ref.		Ref.			Ref.		Ref.	
ESR1/6q25.1	AG	182/162	1.27	(0.97-1.67)	**1.25**	**(0.93-1.69)**	76/72	1.25	(0.82-1.91)	1.22	(0.77-1.92)	154/170	1.27	(0.88-1.81)	1.20	(0.79-1.80)
	AA	34/19	**2.01**	**(1.13-3.68)**	**2.05**	**(1.09-3.97)**	14/8	2.02	(0.83-5.25)	1.90	(0.73-5.25)	106/90	1.96	(0.92-4.37)	2.14	(0.88-5.49)
	Per allele		**1.34**	**(1.08-1.66)**	**1.33**	**(1.05-1.69)**		1.30	(0.93-1.83)	1.34	(0.95-1.91)	20/11	1.32	(1.00-1.76)	1.27	(0.93-1.75)

[a]Alleles on upper line are common alleles; [b]Adjusted for age; [c]Multivariate adjusted for age, BMI, smoking, meat intake, mushroom intake, green and yellow vegetable intake, coffee intake, green tea intake, leisure-time exercise and education. Significant dates are showed in boldface. OR, odds ratio; CI, confidence interval.

Table 3 Age-adjusted odds ratio and multivariate adjusted odds ratio with 95% confidence intervals for lifestyle factors in rs2046210

		Risk allele carriers (AA + AG) n = 474 Case n = 255/Control n = 219							Non-risk allele carriers (GG) n = 457 Case n = 213/Control n = 244						
		n/n	OR[a] (95% CI)		p	OR[b] (95% CI)		p	n/n	OR[a] (95% CI)		p	OR[c] (95% CI)		p
Age (years)		54.0/53.9							55.8/53.2						
Menopausal status	Pre	148/133							130/137						
	Post	107/86							83/107						
Height (cm)	≤150	40/39	1.03	(0.58-1.83)	0.93	0.96	(0.53-1.74)	0.89	55/39	1.34	(0.78-2.9)	0.29	1.19	(0.66-2.14)	0.57
	151-155	76/77	Ref.			Ref.			68/68	Ref.			Ref.		
	156-160	89/66	1.38	(0.88-2.16)	0.16	1.44	(0.91-2.29)	0.12	63/89	0.76	(0.48-1.3)	0.27	0.89	(0.53-1.48)	0.64
	>160	46/34	1.41	(0.81-2.47)	0.23	1.62	(0.91-2.91)	0.10	25/47	0.59	(0.32-1.08)	0.09	0.51	(0.25-0.99)	0.05
BMI (Kg/m^2)	20	59/46	1.27	(0.75-2.14)	0.37	1.13	(0.67-1.94)	0.64	43/50	1.62	(0.93-2.81)	0.09	1.54	(0.84-2.82)	0.16
	20-21.9	69/67	Ref.			Ref.			48/82	Ref.			Ref.		
	22-23.9	58/50	1.09	(0.66-1.80)	0.75	0.97	(0.58-1.63)	0.92	43/52	1.40	(0.82-2.40)	0.22	1.47	(0.83-2.63)	0.19
	≥24	65/53	1.17	(0.71-1.94)	0.53	1.09	(0.65-1.82)	0.74	74/59	**2.07**	**(1.26-3.43)**	**<0.01**	**1.91**	**(1.11-3.29)**	**0.02**
Smoking status	Never	222/201	Ref.			Ref.			180/230	Ref.			Ref.		
	Current or former	29/15	1.78	(0.93-3.51)	0.08	1.61	(0.83-3.21)	0.16	31/13	**3.82**	**(1.94-7.98)**	**<0.01**	**3.86**	**(1.87-8.37)**	**<0.01**
Alcohol drinking	Never	129/107	Ref.			Ref.			108/111	Ref.			Ref.		
	Current or former	125/109	0.97	(0.67-1.40)	0.97	1.07	(0.73-1.57)	0.74	105/133	0.91	(0.62-1.33)	0.61	0.87	(0.56-1.33)	0.51
Alcohol intake (g/day)	0	129/107	Ref.			Ref.			108/111	Ref.			Ref.		
	<5	75/56	1.12	(0.72-1.74)	0.61	1.22	(0.78-1.92)	0.39	64/73	0.99	(0.64-1.54)	0.98	0.98	(0.60-1.61)	0.94
	5-10	28/32	0.75	(0.42-1.34)	0.34	0.88	(0.49-1.60)	0.68	25/30	0.94	(0.51-1.72)	0.85	0.92	(0.46-1.80)	0.80
	10>	20/19	0.88	(0.44-1.74)	0.71	0.94	(0.46-1.89)	0.85	16/26	0.70	(0.35-1.38)	0.31	0.55	(0.24-1.22)	0.14
Leisure-time exercise	No	143/97	Ref.			Ref.			110/116	Ref.			Ref.		
	Yes	110/121	**0.62**	**(0.43-0.89)**	**0.01**	**0.60**	**(0.41-0.87)**	**<0.01**	101/127	0.77	(0.52-1.12)	0.17	0.74	(0.49-1.11)	0.14
Intensity of physical activity[d] (met/week)	0	143/99	Ref.			Ref.			109/119	Ref.			Ref.		
	>6.0	25/23	0.79	(0.42-1.48)	0.45	0.72	(0.38-1.37)	0.32	25/19	1.35	(0.70-2.63)	0.37	1.20	(0.59-2.48)	0.61
	6.0-11.9	20/28	**0.49**	**(0.26-0.92)**	**0.03**	**0.46**	**(0.24-0.86)**	**0.02**	22/32	0.63	(0.34-1.17)	0.15	0.66	(0.34-1.28)	0.22
	12.0-23.9	27/36	**0.52**	**(0.29-0.91)**	**0.02**	**0.53**	**(0.30-0.94)**	**0.03**	21/44	**0.48**	**(0.26-0.85)**	**0.01**	**0.45**	**(0.24-0.83)**	**0.01**
	≥24.0	30/32	0.65	(0.37-1.14)	0.13	0.68	(0.38-1.20)	0.18	22/29	0.74	(0.40-1.38)	0.35	0.70	(0.36-1.36)	0.30
Age at menarche	≤12	70/92	0.73	(0.45-1.19)	0.73	0.72	(0.44-1.19)	0.20	68/109	1.07	(0.63-1.81)	0.80	0.98	(0.56-1.70)	0.93
(year)	13	66/55	Ref.			Ref.			43/58	Ref.			Ref.		
	≤14	116/68	1.20	(0.74-1.93)	1.20	1.15	(0.71-1.89)	0.57	99/75	1.32	(0.78-2.25)	0.29	1.62	(0.93-2.84)	0.09

Table 3 Age-adjusted odds ratio and multivariate adjusted odds ratio with 95% confidence intervals for lifestyle factors in rs2046210 *(Continued)*

Parity	0	54/35	Ref.			Ref.			31/40	Ref.			Ref.		
	1-2	123/122	0.63	(0.38-1.04)	0.07	0.66	(0.40-1.10)	0.11	124/143	0.95	(0.55-1.64)	0.85	1.12	(0.61-2.09)	0.71
	≥3	54/53	0.65	(0.36-1.15)	0.14	0.65	(0.36-1.17)	0.15	46/53	0.94	(0.50-1.76)	0.84	1.29	(0.64-2.62)	0.48
Age at first childbirth	<25	78/68	1.21	(0.77-1.90)	0.40	1.08	(0.68-1.71)	0.74	72/74	1.22	(0.78-1.91)	0.38	1.17	(0.71-1.91)	0.54
(year)	25-29	87/89	Ref.			Ref.			75/97	Ref.			Ref.		
	≥30	33/22	1.55	(0.84-2.90)	0.16	1.45	(0.77-2.76)	0.25	30/28	1.39	(0.77-2.54)	0.27	1.77	(0.92-3.45)	0.09
Breastfeeding	No	72/51	Ref.			Ref.			51/53	Ref.			Ref.		
	Yes	178/165	0.76	(0.50-1.16)	0.21	0.77	(0.50-1.17)	0.22	159/189	0.83	(0.53-1.30)	0.42	1.02	(0.62-1.69)	0.93
Family history of	No	209/180	Ref.			Ref.			178/192	Ref.			Ref.		
Breast cancer	Yes	31/24	1.11	(0.63-1.97)	0.55	1.12	(0.63-2.00)	0.71	22/28	0.82	(0.45-1.50)	0.75	1.07	(0.57-2.05)	0.83
Education	High school or less	135/99	Ref.			Ref.			123/96	Ref.			Ref.		
	Two-year college	81/63	0.93	(0.61-1.42)	0.74	0.95	(0.62-1.47)	0.83	60/81	**0.62**	**(0.40-0.95)**	**0.03**	**0.59**	**(0.37-0.94)**	**0.03**
	University	36/55	**0.48**	**(0.29-0.79)**	**<0.01**	**0.48**	**(0.29-0.79)**	**<0.01**	28/65	**0.35**	**(0.21-0.59)**	**<0.01**	**0.38**	**(0.22-0.66)**	**<0.01**

[a]OR is adjusted for age.
[b]Multivariate adjusted for leisure-time exercise and education.
[c]Multivariate adjusted for BMI, smoking state, intensity of physical activity and education.
[d]Intensity of physical activity and education. Significant dates are showed in boldface.
OR, odds ratio; CI, confidence interval; BMI, body mass index.

Table 4 Age-adjusted odds ratio and multivariate adjusted odds ratio with 95% confidence intervals for lifestyle factors in rs3757318

		Risk allele carriers(AA + AG) n = 397 Case n = 216/Control n = 181							non-risk allele carriers(GG) n = 530 Case n = 249/Control n = 281						
		n/n	OR[a] (95%CI)		p	OR[b] (95% CI)		p	n/n	OR[a] (95% CI)		p	OR[c] (95% CI)		p
Age (years)		54.23/53.30							55.28/53.76						
Menopausal status	Pre	124/101							154/170						
	Post	92/80							95/111						
Height (cm)	≤150	36/28	1.24	(0.66-2.34)	0.50	1.46	(0.68-3.16)	0.33	58/50	1.07	(0.65-1.77)	0.78	1.01	(0.60-1.69)	0.98
	151-155	62/63	Ref.			Ref.			84/80	Ref.			ref.		
	156-160	78/51	1.57	(0.96-260)	0.07	1.57	(0.86-2.90)	0.14	72/105	0.68	(0.44-1.05)	0.08	0.73	(0.47-1.15)	0.18
	>160	36/38	1.00	(0.55-1.80)	0.99	0.58	(0.26-1.24)	0.16	34/43	0.80	(0.46-1.39)	0.43	0.89	(0.50-1.59)	0.70
BMI(Kg/m^2)	<20	48/37	1.36	(0.77-2.40)	0.26	1.11	(0.54-2.29)	0.77	54/59	1.57	(0.95-2.59)	0.06	1.60	(0.95-2.69)	0.08
	20-21.9	59/60	Ref.			Ref.			54/90	Ref.			Ref.		
	22-23.9	47/35	1.35	(0.77-2.40)	0.24	1.57	(0.80-3.12)	0.19	57/66	1.41	(0.86-2.30)	0.40	1.29	(0.78-2.14)	0.32
	≥24	57/48	1.18	(0.69-2.01)	0.51	1.14	(0.60-2.17)	0.68	81/63	**2.08**	**(1.29-3.37)**	**<0.01**	**1.89**	**(1.16-3.10)**	**0.01**
Smoking status	Never	186/168	Ref.			Ref.			214/262	Ref.			Ref.		
	Current or former	25/11	**2.15**	**(1.05-4.71)**	**0.04**	**2.73**	**(1.07-7.65)**	**0.04**	34/17	**2.82**	**(1.53-5.40)**	**<0.01**	**2.39**	**(1.27-4.65)**	**<0.01**
Alcohol drinking	Never	114/90	Ref.			Ref.			124/127	Ref.			Ref.		
	Current or former	101/89	0.93	(0.62-1.39)	0.71	0.99	(0.60-1.65)	0.97	125/153	0.90	(0.63-1.28)	0.55	0.95	(0.65-1.38)	0.78
Alcohol intake	0	114/90	Ref.			Ref.			124/127	Ref.			Ref.		
(g/day)	<5	59/45	1.08	(0.67-1.76)	0.75	1.12	(0.61-2.04)	0.72	78/84	1.01	(0.67-1.51)	0.98	1.11	(0.72-1.70)	0.64
	5-10	27/27	0.81	(0.44-1.49)	0.50	0.88	(0.41-1.90)	0.75	25/35	0.79	(0.44-1.41)	0.43	0.89	(0.49-1.63)	0.71
	10>	13/16	0.65	(0.29-1.41)	0.27	0.78	(0.27-2.14)	0.63	22/29	0.82	(0.44-1.52)	0.54	0.66	(0.33-1.28)	0.22
Leisure-time	No	122/80	Ref.			Ref.			127/133	Ref.			Ref.		
Exercise	Yes	93/101	**0.58**	**(0.39-0.87)**	**<0.01**	0.78	(0.47-1.27)	0.32	119/146	0.82	(0.58-1.17)	0.27	0.84	(0.59-1.21)	0.35
Intensity of physical activity[d](met/week)	0	122/81	Ref.			Ref.			126/137	Ref.			Ref.		
	>6.0	23/17	0.87	(0.44-1.76)	0.70	1.62	(0.68-4.03)	0.28	28/25	1.24	(0.68-2.27)	0.48	1.19	(0.64-2.25)	0.58
	6.0-11.9	21/25	0.55	(0.28-1.04)	0.07	0.58	(0.27-1.21)	0.15	23/34	0.68	(0.37-1.22)	0.20	0.69	(0.37-1.28)	0.24
	12.0-23.9	19/32	**0.39**	**(0.20-0.73)**	**<0.01**	0.73	(0.33-1.56)	0.41	29/48	0.63	(0.37-1.06)	0.08	0.62	(0.36-1.06)	0.08
	≥24.0	23/26	0.56	(0.29-1.06)	0.07	0.67	(0.31-1.42)	0.29	27/35	0.79	(0.45-1.39)	0.42	0.84	(0.47-1.50)	0.55
Age at menarche	≤12	63/73	1.00	(0.59-1.70)	0.99	1.67	(0.85-3.33)	0.14	73/127	0.77	(0.47-1.24)	0.28	0.74	(0.45-1.22)	0.24
(year)	13	52/51	Ref.			Ref.			57/61	Ref.			Ref.		
	≤14	99/56	1.39	(0.82-2.35)	0.22	1.74	(0.90-3.37)	0.10	115/88	1.12	(0.70-1.81)	0.63	1.02	(0.62-1.68)	0.92

Table 4 Age-adjusted odds ratio and multivariate adjusted odds ratio with 95% confidence intervals for lifestyle factors in rs3757318 *(Continued)*

Parity	0	49/24	Ref.			Ref.			37/50	Ref.			Ref.		
	1-2	110/105	**0.48**	**(0.27-0.84)**	**<0.01**	0.55	(0.19-1.54)	0.25	132/160	0.98	(0.60-1.62)	0.95	1.19	(0.70-2.05)	0.52
	≥3	36/48	**0.34**	**(0.17-0.65)**	**<0.01**	0.35	(0.12-1.04)	0.06	65/58	1.36	(0.77-2.40)	0.29	1.74	(0.95-3.21)	0.07
Age at first childbirth	<25	60/60	1.05	(0.64-1.71)	0.86	0.97	(0.56-1.66)	0.90	88/82	1.35	(0.89-2.05)	0.15	1.19	(0.77-1.84)	0.43
(year)	25-29	72/77							88/110	Ref.			Ref.		
	≥30	34/19	**1.96**	**(1.03-3.80)**	**0.04**	1.82	(0.88-3.85)	0.11	29/31	1.17	(0.66-2.10)	0.59	1.27	(0.69-2.33)	0.45
Breastfeeding	No	65/38	Ref.			Ref.			59/65	Ref.			Ref.		
	Yes	150/143	**0.60**	**(0.38-0.95)**	**0.03**	0.93	(0.36-2.43)	0.89	183/211	0.91	(0.61-1.38)	0.67	1.07	(0.69-1.65)	0.77
Family history of	No	173/143	Ref.			Ref.			212/229	Ref.			Ref.		
Breast cancer	Yes	24/19	1.04	(0.55-2.00)	0.79	1.30	(0.56-3.07)	0.54	28/33	0.91	(0.53-1.57)	0.93	0.90	(0.51-1.58)	0.72
Education	High school or less	113/80	Ref.			Ref.			144/115	Ref.			Ref.		
	Two-year college	74/54	0.99	(0.62-1.57)	0.96	1.02	(0.58-1.79)	0.94	66/90	**0.60**	**(0.40-0.91)**	**0.01**	**0.63**	**(0.42-0.96)**	**0.03**
	University	27/45	**0.43**	**(0.24-0.76)**	**<0.01**	**0.33**	**(0.16-0.67)**	**0.00**	36/74	**0.40**	**(0.25-0.64)**	**<0.01**	**0.45**	**(0.28-0.73)**	**<0.01**

[a]OR is adjusted for age.
[b]Multivariate adjusted for smoking state, leisure-time exercise, party, age of first children, breastfeeding and education. [c]Multivariate adjusted for BMI, smoking state, and education. [d]Intensity of physical activity and education. Significant dates are showed in boldface. OR, odds ratio; CI, confidence interval; BMI, body mass index.

located 200 kb upstream of ESR1. The risk allele frequency of rs3757318 is 6.6% in Europeans (HapMap-CEU), 33% in Chinese (HapMap-HCB) and 25% in Japanese (HapMap-JTP) [19]. We found a 22% risk allele frequency, consistent with HapMap-JTP. Thus, the risk allele frequency for rs3757318 varies between Europeans and Asians. In an analysis of the association between rs2046210 and rs12662670 as a surrogate for rs3757318 and breast cancer risk, Heins et al. found that that per allele OR for rs3757318 was higher in Asians (1.29, 95% CI 1.19–1.41) than in Europeans (1.12, 95% CI 1.08–1.17) [31]. These results suggest that screening for the rs3757318 genotype may be important in Asian women.

We also found that SNPs associated with breast cancer differed with regard to menses state, with rs2046210 and rs3803662 associated with breast cancer risk in premenopausal women. rs3803662 lies 8 kb upstream of TNRC9 and was found to have a significant association with breast cancer risk by Easton et al. [12]. TNRC9 is located on chromosome 16q12 and consists of seven exons. The protein encoded by this gene is a member of the high mobility group box (HMG-box) family. TNRC9 is expressed in brain and breast tissue, and has a higher expression level in breast cancer compared to that in normal tissue [37]. The risk allele frequency of rs3803662 is 24% in Europeans (HapMap-CEU), 72% in Chinese (HapMap-HCB) and 60% in Japanese (HapMap-JTP) [19]. Thus, Asian populations have a higher risk allele frequency than Europeans. However, Chen et al. found that rs3803662 was significantly associated with breast cancer in Europeans [17], but that this relationship was unclear in Asians [38]. Among the breast cancer-associated SNPs found in the current study, rs2046210 and rs3757318 are located near ESR1 and are related to breast cancer risk in Asians. To examine whether lifestyle factors associated with breast cancer risk vary in risk allele and non-risk allele carriers, we performed a subgroup analysis. Leisure-time exercise were associated with a decreased breast cancer risk in rs2046210 risk allele carriers. Although low-penetrance susceptibility SNPs may confer only a small effect on breast cancer risk alone, the risk for development of breast cancer in a risk allele carrier is about 1.2-1.3 fold higher than that in non-carriers. However, our results suggest that risk allele carriers can reduce their breast cancer risk through exercise, whereas obesity and smoking may increase breast cancer risk in non risk-allele carriers. An understanding of the mechanisms underlying the different lifestyle factors associated with breast cancer in rs2046210 and rs3757318 risk allele and non-risk allele carriers may clarify the effects of these SNPs located near ESR1. Examination of interactions between SNPs and lifestyle factors in a larger Japanese population is needed to confirm the current findings for SNPs, lifestyle factors and breast cancer.

Conclusions

This case–control study showed that rs2046210 and rs3757318 located near the ESR1 gene and rs3808662 located on TNRC9 are associated with breast cancer risk in Japanese women. Our results suggest that leisure-time exercise can reduce the breast cancer risk in rs2046210 risk allele carriers, whereas smoking and obesity may increase the breast cancer risk in non-risk allele carriers. Further studies are required to confirm the validity of the association of these SNPs and lifestyle factors with breast cancer risk in the Japanese population.

Abbreviations

SNPs: Single nucleotide polymorphisms; WCRF/AICR: World Cancer Research Fund/American Institute for Cancer Research; NIC: National Cancer Institute; GWAS: Genome-wide association studies; LD: Linkage disequilibrium; BMI: Body mass index; MET: Metabolic equivalent; OR: Odds ratio; CI: Confidence interval; ERα: estrogen receptor α.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

NT designed the study. TM carried out genotyping, performed statistical analysis, and wrote the manuscript with NT. KN participated in genotyping and statistical analysis. TN, TI, TM, TS, JM, HD, SI, HK, KK, YI and YO obtained informed consent from subjects, collected blood samples and data from subjects, and provided advice on the study. YK designed the study and served as an advisor. All authors read and approved the final manuscript.

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Extended trastuzumab therapy improves the survival of HER2-positive breast cancer patients following surgery and radiotherapy for brain metastases

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Abstract. Brain metastases usually present late during the course of breast cancer and are associated with an unfavorable prognosis. It was previously demonstrated that the status of estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor type 2 (HER2) may be altered in the time window between the emergence of the primary breast tumor and the development of metastases. The aim of this study was to compare the expression of ER, PR and HER2 in pathology samples of primary breast cancer and brain metastases in order to evaluate whether previously administered therapy was able to modify this status and determine whether biomarker alterations affect prognosis after the development of brain metastases. Data were collected from 62 patients who were initially diagnosed with breast cancer that had metastasized to the brain. The ER, PR and HER2 status of the samples from the primary tumors and the brain metastases was determined. Differences in the immunohistochemical profiles of ER, PR or HER2 between the primary tumors and the brain metastases in 17 patients (29.3%) were identified. The patients with HER2-positive brain metastases who received trastuzumab had no leptomeningeal metastases and exhibited a longer survival time after brain metastases compared to the HER2-positive patients who did not receive trastuzumab and the patients with HER2-negative brain metastases (P=0.0005). Our results suggested that the patients treated with trastuzumab following surgery and radiotherapy for brain metastases exhibited a better prognosis. Thus, the HER2 status in brain metastases requires re-evaluation and extended trastuzumab therapy is recommended after brain metastases.

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Key words: breast cancer, brain metastases, HER2, trastuzumab, blood-brain barrier

Introduction

Brain metastases, including leptomeningeal metastases (LMM), usually present late during the course of breast cancer and are associated with an unfavorable prognosis. Several previous studies demonstrated that the status of estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor type 2 (HER2) is altered at a certain point between the emergence of the primary breast tumor and the development of metastases (1-5). The mechanism of this discordance in ER, PR and HER2 status between primary tumors and metastases has not been fully elucidated. It was previously reported that the majority of the tumors that do initially respond to targeted therapies may eventually develop acquired resistance (6). Other possible mechanisms are a genetic drift occurring during tumor progression (7) or intratumoral heterogeneity, wherein the clone with the more aggressive phenotype initiates the micrometastatic process (8,9). The number of available pathoanatomical studies on the brain-metastasizing type of breast cancer that evaluated the extent to which the hormone and HER2 receptor discordance between paired pathology samples of primary and metastatic breast cancer specimens affect the prognosis is limited (1-5). Furthermore, few studies reported the biological marker alterations between primary tumors and brain metastases (5,10,11). Post-operative adjuvant treatment decisions are commonly based on the expression of ER, PR and HER2 of

primary tumors. Moreover, treatment decisions for recurrent brain tumor cases are generally based on the ER and HER2 status of the primary tumors. Trastuzumab is a humanized monoclonal antibody directed against the HER2/neu oncoprotein and has the ability to inhibit tumor growth in breast cancer patients overexpressing HER2 (12). However, the pharmacokinetics and effect of trastuzumab on the brain after brain metastases have not been determined. The aim of this study was to compare the expression of ER, PR and HER2 in pathology samples from primary tumors and brain metastases in order to evaluate whether the previous therapy was able to modify this status and to determine whether biomarker alterations affect prognosis after brain metastases. We also investigated the effect of trastuzumab therapy after brain metastases.

Materials and methods

Patients and tissue samples. Data were collected from 62 patients who were initially diagnosed with breast cancer and underwent surgical removal of brain metastases between 2000 and 2012 at the Osaka Medical Center for Cancer and Cardiovascular Diseases and the National Cancer Center Hospital, Japan. These patients had received treatment for primary breast cancer between 1983 and 2011 and undergone surgery for primary breast cancer and brain metastases. Tumor samples were collected from the primary resected breast cancer and metastatic brain lesions. However, not all the primary tumor samples acquired during operation at other hospitals were obtained. We only evaluated specimens considered sufficient for ER, PR and HER2 status estimation.

The ER, PR and HER2 status was determined in the samples from the primary and metastatic lesions. The first brain metastatic-free survival time was defined as the time from the first surgery for the primary tumor to the first detection of brain metastasis on magnetic resonance imaging (MRI). The second brain metastatic-free survival time was defined as the time from the first surgery for brain lesions to the second occurrence of brain metastases on MRI or patient death from any cause. LMM was diagnosed by radiological findings or the cytological evaluation of cerebrospinal fluid obtained by lumbar puncture. Detailed information on the 62 patients is provided in Table I.

This study was approved by the Institutional Review Board of each center.

Histopathological analysis. Surgical specimens were fixed in 10% formalin and embedded in paraffin. Hematoxylin and eosin-stained specimens were examined in order to determine the histological tumor type. Multiple serial sections were subjected to immunohistochemical analysis to assess local staining. Furthermore, tissue sections were subjected to 15 min of microwave heating to activate antigens in a retrieval solution consisting of 0.1 mol/l sodium citrate (pH 6.0), followed by immunostaining of the specimens with the streptavidin-biotin-peroxidase complex method (Vectastain; Vector Laboratories, Burlingame, CA, USA). Human monoclonal antibodies were used against ER (clone 1D5; Dako, Carpinteria, CA, USA) or PR (clone PgR636; Dako) with the streptavidin-biotin method and were considered positive if ≥10% of the nuclei in the invasive component of the tumor were stained (13,14). The HER2/neu status, as assessed using the HercepTest assay (Dako), was scored by the pathologists at each center on a scale of 0 to 3+, according to the Dako scoring system. HER2/neu positivity was defined as HER2/neu 3+ or HER2/neu 2+ and fluorescence *in situ* hybridization positivity.

Table I. Characteristics of patients with brain metastases from breast cancer.

Characteristics	Patient no.	Years	%
Gender			
Female	59		95.2
Male	3		4.8
Age at onset			
Median		45.5	
Range		31-76	
Age at first brain metastases			
Median		51	
Range		35-79	
RPA classification			
Class 1	14		22.6
Class 2	40		64.5
Class 3	8		12.9
Radiotherapy			
WBRT	34		54.8
WBRT + LBRT	3		4.8
WBRT + SRS	13		21.0
LBRT	9		14.5
LBRT + SRS	1		1.6
None	2		3.2
Second BM			
Local and distant	26		41.9
LMM	10		16.1
No second recurrence	22		35.5
Unknown	4		6.5
Median overall survival		6.5	
Median survival time after BM		1.1	
Median first BM-free survival		4.0	
Median second brain BM-free survival		0.6	

RPA, recursive partitioning analysis; WBRT, whole-brain radiotherapy; LBRT, local brain radiotherapy; SRS, stereotactic radiosurgery; LMM, leptomeningeal metastases; BM, brain metastases.

Statistical analysis. Metastatic-free survival and overall survival (OS) times were calculated with the Kaplan-Meier method and differences between groups were compared using

Table II. Alterations in ER and HER2 status in primary tumor and brain metastases.

Primary breast cancer ER/HER2 status	Brain metastases ER/HER2 status			
	(+/+)	(+/-)	(-/+)	(-/-)
(+/+)	0 (0%)	0 (0%)	2 (3.4%)	0 (0%)
(+/-)	0 (0%)	2 (3.4%)	0 (0%)	3 (5.2%)
(-/+)	1 (1.7%)	1 (1.7%)	18 (31.0%)	2 (3.4%)
(-/-)	3 (5.2%)	3 (5.2%)	1 (1.7%)	22 (37.9%)

ER, estrogen receptor; HER2, human epidermal growth factor receptor type 2.

the log-rank test (JMP software version 8; SAS Institute Inc., Cary, NC, USA).

Results

Metastatic-free survival and OS time. The 62 patients underwent resections of the first brain metastases. The median age at the first brain metastasis was 51 years. Thirty-four patients received whole-brain radiotherapy (WBRT). Three patients received WBRT and local brain radiotherapy (LBRT) and 13 patients received WBRT and stereotactic radiosurgery (SRS). Nine patients received LBRT and one received LBRT plus SRS. Two patients were observed without radiotherapy following surgical resection of the first brain metastases (Table I). Five of the nine patients who only received LBRT developed a second brain recurrence negative for LMM whereas the remaining four patients did not develop second brain metastases.

The median first and second brain metastatic-free survival times, the survival time after brain metastases and the OS time from the initial diagnosis of breast cancer for the 62 breast cancer patients were 4.0, 0.6, 1.1 and 6.5 years, respectively (Table I). The 5-year OS rate from surgical resection of brain metastases was 11.1%. Patients with recursive partitioning analysis (RPA) (15) class I and II had a more favorable OS compared to class III patients, although RPA classes did not significantly differ in survival time after brain metastases (Fig. 1, P=0.16).

Alteration of ER, PR and HER2 status in primary tumor and brain metastases. The alterations in the ER and HER2 status in primary tumors and brain metastases are presented in Table II. The positive rate of immunohistochemical profiles of ER, PR and HER2 in the primary tumors were 11.7% (7/60), 8.6% (5/58) and 41.4% (24/58), respectively. The positive rates of immunohistochemical profiles of ER, PR and HER2 in brain metastases were 16.1% (10/62), 11.3% (7/62) and 43.5% (27/62), respectively. The rates of immunohistochemical alteration of ER, PR and HER2 and triple-negative status between primary and brain metastases were 21.7% (13/60), 10.3% (6/58), 12.1% (7/58) and 13.8% (8/58), respectively. The immunohistochemical profiles for ER, PR and HER2 differed between the primary tumors and the brain metastases in 17 patients (29.3%; 17/58, Table III). The discordance rates in the 17 patients were 76.5% for ER, 35.3% for PR and 41.2% for

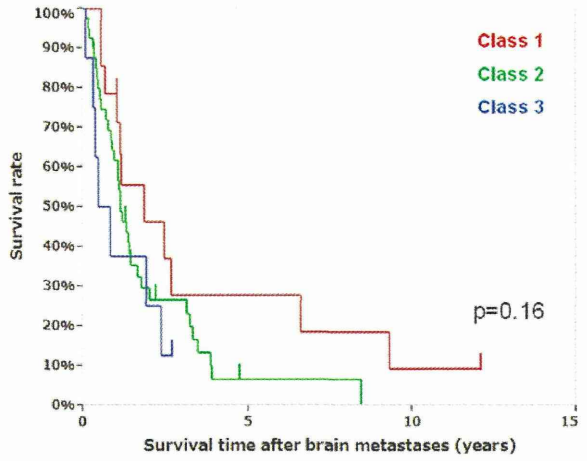

Figure 1. Overall survival from brain metastases by recursive partitioning analysis (RPA) classification. Kaplan-Meier survival curves according to RPA classification after brain metastases.

HER2 (Table III). All the patients with ER or PR alterations (positive-negative or negative-positive) had received hormone therapy prior to the development of brain metastases. Two of three patients with HER2 alteration (positive-negative) had received trastuzumab therapy prior to the development of brain metastases. A HER2-positive status was maintained in 88.9% (16/18) of the patients who had received trastuzumab prior to the development of brain metastases. All the patients with negative-positive alteration in HER2 had received hormone therapy but not trastuzumab prior to the development of brain metastases (Table III). One of five patients (20.0%) with triple-negative breast cancer (TNBC) did not exhibit any status alterations in the brain metastases and had received chemotherapy prior to the development of brain metastases.

Clinical outcome with trastuzumab therapy after brain metastases. Trastuzumab was administered to 18 of 24 patients who had HER2-positive primary tumors prior to brain metastases and to 10 of these 24 patients after brain metastases. Two patients were started on trastuzumab after brain metastases.

The median first brain metastatic-free survival time of patients with a positive HER2 status in the primary tumors with (n=18) and without trastuzumab (n=6) was 4.2 and

Table III. Discordance cases of immunohistochemical profiles between primary tumors and brain metastases.

Case no.	HER2		ER		PR		Chemotherapy prior to BM	Hormone therapy prior to BM	Trastuzumab therapy prior to BM
	Primary	BM	Primary	BM	Primary	BM			
1	-	-	-	+	-	-	-	+	-
2	-	-	-	+	-	-	-	+	-
3	-	-	-	+	-	+	-	+	-
4	-	+	-	-	-	-	+	+	-
5	-	+	-	+	-	-	-	+	-
6	-	+	-	+	-	+	-	+	-
7	-	+	-	+	-	+	-	+	-
8	-	-	+	+	-	+	+	+	-
9	-	-	+	-	+	-	-	+	-
10	-	-	+	-	+	+	-	+	-
11	-	-	+	-	+	+	-	+	-
12	+	-	-	-	-	-	+	-	-
13	+	-	-	-	-	-	+	-	+
14	+	-	-	+	-	-	-	+	+
15	+	+	-	+	-	-	-	+	-
16	+	+	+	-	-	-	+	+	+
17	+	+	+	-	+	-	+	+	-

HER2, human epidermal growth factor receptor type 2; ER, estrogen receptor; PR, progesterone receptor; BM, brain metastases.

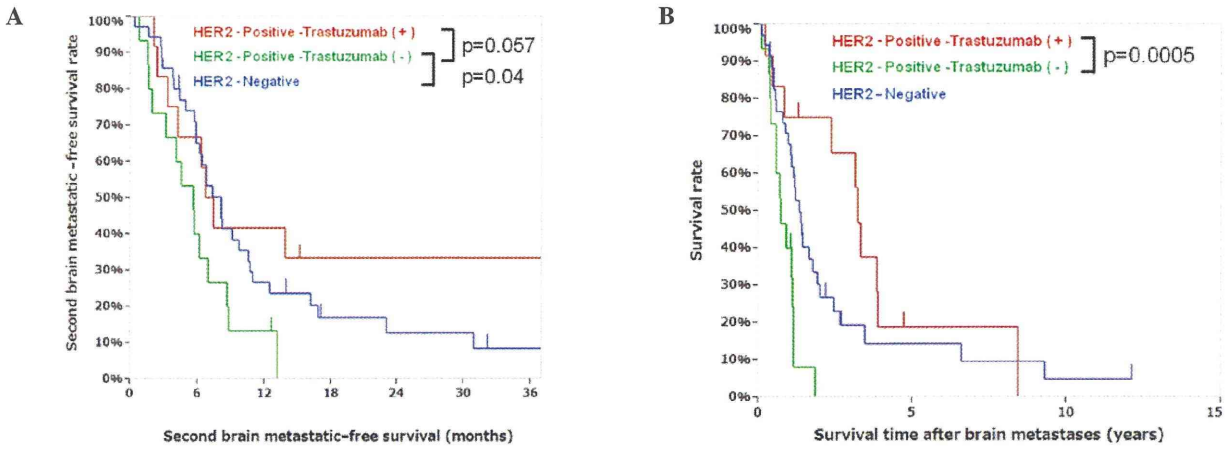

Figure 2. (A) Second brain metastatic-free survival time according to HER2 status and trastuzumab therapy after brain metastases. (B) Overall survival from brain metastases by HER2 status and trastuzumab therapy after brain metastases.

5.3 years, respectively, whereas that of patients with negative HER2 status (n=34) was 4.0 years. The HER2 status in primary tumors with trastuzumab therapy prior to brain metastases did not correlate with the first brain metastatic-free survival time. The second brain metastatic-free survival time of patients with positive HER2 status in brain lesions with (n=12) and without trastuzumab (n=15) was 7.0 and 5.6 months, respectively (P=0.057), whereas that of patients with negative HER2 status (n=35) was 8.0 months (Fig. 2A). The median OS from the first brain metastasis in patients with positive HER2 status with and without trastuzumab was 3.2 and 0.7 years, respectively (P=0.0005, Fig. 2B, Table IV). The HER2 status in brain metastases with trastuzumab therapy after brain metastases did not correlate with the second brain metastatic-free survival time. Compared to HER2-positive patients who did not receive trastuzumab and those with HER2-negative brain metastases, the patients who had HER2-positive brain metastases and received trastuzumab tended to have longer second brain metastatic-free survival times (P=0.057) and exhibited significantly longer survival times after brain metastases

Table IV. Characteristics and survival time according to HER2 status and trastuzumab therapy after brain metastases.

Characteristics	Positive HER2 with trastuzumab	Positive HER2 without trastuzumab	Negative HER2
Patient no.	12	15	35
Median age at first brain metastasis, years (range)	47 (35-67)	54 (39-64)	53 (35-79)
RPA class 1/2/3	0/10/2	4/9/2	10/21/4
ER-brain metastases +/-	0/12	4/11	6/29
PR-brain metastases +/-	1/11	2/13	4/31
Any 2nd brain metastases including LMM	8 (67%)	7 (47%)	21 (60%)
(within 6 months after surgery)	3 (25%)	5 (33%)	8 (23%)
(within 12 months after surgery)	6 (50%)	7 (47%)	17 (49%)
LMM	0 (0%)	4 (27%)	6 (17%)
(within 6 months after surgery)	0 (0%)	4 (27%)	4 (11%)
2nd brain metastatic-free survival time, years (95% CI)	0.6 (0.2-3.3)	0.5 (0.1-0.6)	0.7 (0.5-0.9)
Survival time after brain metastases, years (95% CI)	3.2 (0.4-3.9)	0.7 (0.3-1.1)	1.3 (0.9-1.8)

HER2, human epidermal growth factor receptor type 2; RPA, recursive partitioning analysis; ER, estrogen receptor; PR, progesterone receptor; LMM, leptomeningeal metastases; CI, confidence interval.

(P=0.0005) (Fig. 2A and B, Table IV). Patient characteristics, including age at brain metastasis and proportion of RPA classes, were not different in the HER2-positive group with or without trastuzumab (Table IV). The ER, PR and HER2 status of brain metastatic lesions exhibited no correlation with survival time after brain metastases. There was no difference in survival time after brain metastases between TNBC and HER2-positive patients.

Trastuzumab therapy and postoperative LMM. The incidence of LMM after surgery for brain metastases was 27% (4/15) in patients with HER2-positive central nervous system (CNS) samples who did not receive trastuzumab and 17% (6/35) in patients with HER2-negative CNS samples. All the patients with LMM exhibited neuronal death. Of five patients with recurrent brain metastases who did not receive trastuzumab, four patients (80%) developed LMM within 6 months after surgery for brain metastases. However, none of the 12 patients who were administered trastuzumab after surgery presented with LMM. Thus, trastuzumab therapy after brain metastases decreased the incidence of postoperative LMM (Chi-square test, P=0.053).

Discussion

Breast cancer is the second most common cause of brain metastases. Brain metastases occur in 14-20% of breast cancer patients and usually occur late in the progression of metastatic disease (16). The results of the present study have demonstrated that, among patients with HER2-positive brain metastases, those who received trastuzumab had longer survival times following surgery for brain metastases, compared to those without trastuzumab treatment.

The prolongation of the OS time in HER2-positive breast cancer patients with trastuzumab may be attributed to the decrease in LMM and neuronal death and the effects of trastuzumab on the control of systemic metastases (17-21). A positive HER2 status in primary breast cancer was considered a risk factor for the development of brain metastases (22). Although trastuzumab does not cross the blood-brain barrier (BBB) and has no direct activity on brain metastases, previous studies reported a survival benefit with trastuzumab in HER2-positive patients with brain metastases, who had a significantly longer survival compared to that of HER2-negative patients (17-21), which was consistent with our findings. Trastuzumab therapy prior to brain metastases did not correlate with the first brain metastatic-free survival time in our study, since trastuzumab therapy was not associated with an increased incidence of brain metastases (23,24). The patients with HER2-positive brain metastases who received trastuzumab exhibited longer OS after brain metastases compared to the patients with HER2-positive brain metastases without trastuzumab treatment in our study. The development of LMM was a rare manifestation encountered in 5-8.1% of patients with HER2-positive primary tumors (17,20). None of the patients who received trastuzumab therapy after surgery for brain metastases presented with LMM in our study. Of note, radiotherapy with doses of 20-30 Gy with a fraction size of 2 Gy was reported to increase the permeability of the BBB and may enhance the effect of chemotherapy (25). Thus, surgery followed by WBRT may disrupt the BBB and facilitate the delivery of trastuzumab to the brain in HER2-positive patients.

Hormone receptor and HER2 status are important predictive markers in breast cancer. ER negativity was associated with an increased risk of brain metastases (26-28) and HER2

amplification/overexpression was shown to be a prognostic and predictive factor for the development of brain metastases (29). Approximately two-thirds of early breast cancer patients were found to be ER-positive (30) and the HER2-positivity rate in early breast cancer was reported to be ~15% by a previous study (31). In our study, the ER and HER2-positivity rate in primary tumors was lower (11.7%) and higher (41.4%), respectively, compared to those reported by previous studies on primary breast tumors.

Several previous studies demonstrated that the discordance in biomarker expression between primary tumors and metastases and the alteration of hormone receptor and HER2 status is affected by adjuvant chemotherapy associated with hormone therapy and may affect the prognosis (1-5,32,33). The alterations were possibly attributed to a genetic drift or clonal selection during tumor progression (7) or to the presence of intratumoral heterogeneity in ER, PR and HER2 status (8,9). In previous studies that investigated primary breast cancer and distant metastases, the locoregional recurrence or lymph node metastasis, including brain metastasis, exhibited discordance rates of 0-37.5% (34-40). In our study, the immunohistochemical profiles for ER, PR and HER2 differed between the primary tumors and the brain metastases in 29.3% of the patients. Prior hormone therapy or chemotherapy exerted an effect on this discordance phenomenon (1-5,32,33). All the patients with ER or PR alterations (positive-negative and negative-positive) had received hormone therapy prior to the development of brain metastases. Negative-positive alterations were observed in 15.1% of ER-negative, 7.5% of PR-negative and 11.8% of HER2-negative primary tumors in our study. The HER2 status was highly maintained and the concordance rate between primary tumors and systemic metastases was shown to be 97% by a previous study (41). We demonstrated that 89% of HER2-positive patients treated with trastuzumab prior to the development of brain metastases maintained a positive status in brain metastases. The possibility of the discordance rate in HER2 expression between primary and metastatic tumors was less frequent compared to ER or PR (10,11,34-40,42); however, the possibility of intratumoral heterogeneity must be considered, with re-evaluation of the HER2 status in brain metastases for further systemic treatment after brain metastases. New HER2-targeted drugs, such as lapatinib, are able to cross the BBB and thereby control brain metastases and other systemic breast cancer metastases more effectively (43).

In conclusion, 11.8% of HER2-negative patients with primary breast cancers had positive HER2 status alterations in the brain metastases. Patients treated with trastuzumab after surgery for brain metastases and radiotherapy exhibited a better prognosis. Thus, the HER2 status in brain metastases requires re-evaluation and the administration of trastuzumab or lapatinib in HER2-positive patients should be considered even after brain metastases.

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ORIGINAL ARTICLE

RASSF1A methylation indicates a poor prognosis in hepatoblastoma patients

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Abstract
Purpose The RAS association domain family protein 1 (RASSF1A) is known to be frequently inactivated by promoter hypermethylation in cancers. This study investigated the association of *RASSF1A* methylation with clinical outcomes in hepatoblastoma patients and whether it is correlated with the histological phenotype of hepatoblastoma tumors.
Methods Seventy-four hepatoblastoma tumors were obtained from patients enrolled in the Japanese study group for pediatric liver tumor protocol-2. From nine formalin-fixed, paraffin-embedded specimens, we extracted DNA by dissection under a light microscope. We examined the methylation status of the *RASSF1A* promoter region by bisulfite pyrosequencing.
Results Twenty-five (33.8 %) hepatoblastoma tumors were classified as having methylated *RASSF1A*. The *RASSF1A* methylation was significantly associated with metastatic tumors and a poor prognosis. Despite the complete resection, five pretreatment extent of disease II tumors showed recurrence or distant metastasis postoperatively. Among these cases, four tumors were found to show *RASSF1A* methylation. When compared to histologically different types of cell, *RASSF1A* methylation values in samples of the normal liver, fetal type, and embryonal type, were significantly elevated in ascending order.
Conclusions We confirmed that *RASSF1A* methylation is a significant prognostic indicator in hepatoblastomas, and it may become a promising molecular marker to stratify patients into appropriate risk groups.

Keywords Hepatoblastoma · *RASSF1A* methylation · Prognostic marker

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Introduction

Hepatoblastoma is the most common malignant neoplasm of the liver in children. Despite the progress of therapy, the mortality rate remains at 35–50 % in high-risk patients, such as those with extrahepatic tumors, macroscopic invasion of large vessels, or distant or lymph node metastases [1]. Complete surgical resection or liver transplantation and mainstream treatment with cytotoxic drugs are essential for achieving a favorable long-term outcome. To

improve the mortality of hepatoblastoma patients in advanced stages, innovative treatment and potent prognostic markers for better therapy planning are needed.

Histologically, hepatoblastoma tumors are classified as wholly epithelial, or mixed epithelial and mesenchymal types. In the wholly epithelial type, there are two major subtypes, the fetal subtype and the mixed fetal and embryonal subtype [2]. Fetal and embryonal components often develop in combination, that is, heterogeneity is present. The RAS association domain family protein 1 (RASSF1A) is known to be frequently inactivated by promoter hypermethylation in many adult and childhood cancers [3]. We previously reported that *RASSF1A* methylation was correlated with a poor outcome by multivariate analysis, and suggested that *RASSF1A* may be a promising molecular–genetic marker predicting the treatment outcome in hepatoblastoma patients [4]. The association between the histological type and *RASSF1A* methylation is ambiguous despite the fact that the histologic features are associated with different prognoses; a pure fetal histology is favorable and small cell undifferentiated and macrotrabecular histologies are unfavorable [1]. Therefore, the current study was undertaken to determine the association with histological types by examining each type of hepatoblastoma cell dissected separately.

In this study, we investigated the methylation status of *RASSF1A* in hepatoblastoma tumors by bisulfite pyrosequencing, which is a rapid and accurate method to quantify DNA methylation. We analyzed the results with regard to patients' clinicopathological characteristics and prognosis, and evaluated its association with the histological phenotype on the basis of the epigenetic alteration of hepatoblastomas.

Methods

Patients and samples

Seventy-four hepatoblastoma patients with a median age of 18 months underwent tumor resection and partial hepatectomy between December 1999 and December 2008 at the institutions of the Japanese Study Group for Pediatric liver Tumors (JPLT). All patients were treated in the JPLT-2 study [5]. The extent of disease was determined at the time of initial biopsy or resection according to the classification of the pretreatment extent of disease (PRETEXT) staging system [6]. Metastatic tumors were found in 15 % of the patients (Table 1). The 5-year overall survival and event-free survival rates were 86.7 and 73.4 % for the 74 patients, respectively.

The DNA samples of the 74 hepatoblastoma tumors were supplied by JPLT, and they were extracted from fresh-frozen specimens. Furthermore, formalin-fixed, paraffin-embedded (FFPE) specimens were obtained from nine patients referred to our institution for surgical treatment between 1995 and 2011. We extracted DNA from different types of cell: fetal type, embryonal type, and normal liver, by dissection under a light microscope in order to avoid contamination with normal tissues and mesenchymal components. The ethics committee of our institution approved the study protocol, and signed

Table 1 Clinicopathological factors and *RASSFIA* methylation status in 74 patients with hepatoblastoma

Clinicopathological factors		No. of patients	*RASSF1A*		p value[1]
			Methylated	Unmethylated	
Sex	Male	45	14	31	0.360
	Female	29	11	18	
Age at diagnosis	<365 days	22	0	22	0.000064
	≥365 days	52	25	27	
PRETEXT	I	5	1	4	0.319
	II	27	7	20	
	III	29	10	19	
	IV	13	7	6	
Metastasis	No	63	15	48	0.000039
	Yes	11	10	1	
Histological type	Fetal	28	9	19	0.508
	Mixed fetal and embryonal	40	14	26	
	Unknown	6			
Outcome	Alive	63	15	48	0.000039
	Dead	11	10	1	

[1] Fisher's exact test

informed consent was obtained in all cases by local physicians of the participating institutions.

Evaluation of *RASSF1A* methylation level

We examined the methylation status of the *RASSF1A* promoter region by bisulfite pyrosequencing, which can calculate the level of methylation at each CpG site in samples after bisulfite treatment. Genomic DNA (500 ng) was modified with sodium bisulfite using an EpiTect bisulfite kit (Qiagen, Netherlands). Bisulfite pyrosequencing was carried out as described previously [7]. After PCR, the biotinylated PCR product was purified, made single-stranded, and used as a template in the pyrosequencing reaction. Briefly, the PCR products were bound to streptavidin sepharose beads HP (Amersham Biosciences, USA), after which beads containing the immobilized PCR product were purified, washed, and denatured using a 0.2 mol/L NaOH solution. After the addition of 0.3 μmol/L sequencing primer to the purified PCR product, pyrosequencing was carried out using a PSQ96MA system (Biotaga) and Pyro Q-CpG software (Biotage). The mean value of the methylation levels at two CpG sites in the *RASSF1A* promoter region was calculated. Primer sequences used in this study were as follows: forward, GAAGGAGGGAAGGAAGGGTAAG; reverse, GCCTCC CCCAAAATCCAA; sequencing primer, TTGTATTTAG GTTTTTATTG.

Statistical analysis

Correlations between the *RASSF1A* methylation status and clinicopathological factors were analyzed using the Fisher's exact test. Survival curves were constructed according to the methods of Kaplan and Meier, and comparisons of survival curves were performed with a log-rank test. One-way ANOVA followed by Student's *t* test with Bonferroni correction was used to compare methylation values of histologically different types of cell. A P value <0.05 was considered statistically significant.

Results

RASSF1A methylation status in 74 hepatoblastomas

The average of the *RASSF1A* methylation values in 74 hepatoblastoma tumors was 25.8 % (2.0–74.8 %). We performed the ROC analysis to determine the cutoff value of the *RASSF1A* methylation and adopted a cutoff value of 36.2 % in this study (Fig. 1). On the basis of this cutoff value, 25 (33.8 %) tumors were classified as having methylated *RASSF1A*, and the sensitivity and specificity for

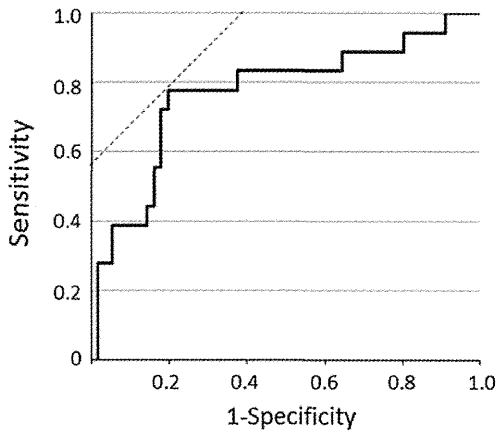

Fig. 1 ROC analysis to determine the cutoff value of the *RASSF1A* methylation

the patients having an event postoperatively were 77.8 and 80.4 %, respectively. There was only one patient who died in those with tumors with *RASSF1A* unmethylated (Fig. 2).

Associations between clinicopathological factors and *RASSF1A* methylation status

We evaluated the associations between the clinicopathological factors and *RASSF1A* methylation status in 74 patients. As Table 1 shows, there were no patients aged under 1 year who had a tumor with *RASSF1A* methylated; however, about half of the patients aged over 1 year were found to have a tumor with *RASSF1A* methylated. 10 of 25 patients (40 %) with a tumor with *RASSF1A* methylated suffered from metastatic tumors, although there was only one patient with metastasis in those with a tumor with *RASSF1A* unmethylated. This demonstrated that age at diagnosis and metastatic tumors were significantly associated with *RASSF1A* methylation. In Kaplan–Meier analyses, the patients with a tumor with methylated *RASSF1A* were significantly associated with a poor outcome: the 5-year overall survival and event-free survival rates were 63.6 and 35.5 %, respectively (Fig. 3).

The *RASSF1A* methylation was detected in 1 of 5 PRETEXT I tumors and 7 of 27 PRETEXT II tumors (Table 1). Despite complete resection, five PRETEXT II tumors showed recurrence or distant metastasis postoperatively, and three patients died. Among these cases, four tumors were found to have *RASSF1A* methylated.

Associations between histological types and *RASSF1A* methylation status

Next, we evaluated whether *RASSF1A* methylation is associated with the histopathological subtypes. Four of the

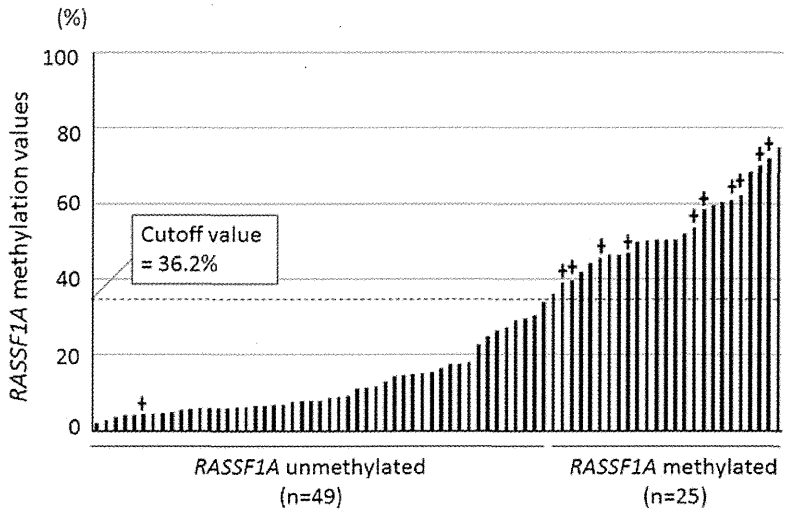

Fig. 2 *RASSF1A* methylation values in 74 patients with hepatoblastoma. *Plus* indicates the patient who died of the disease

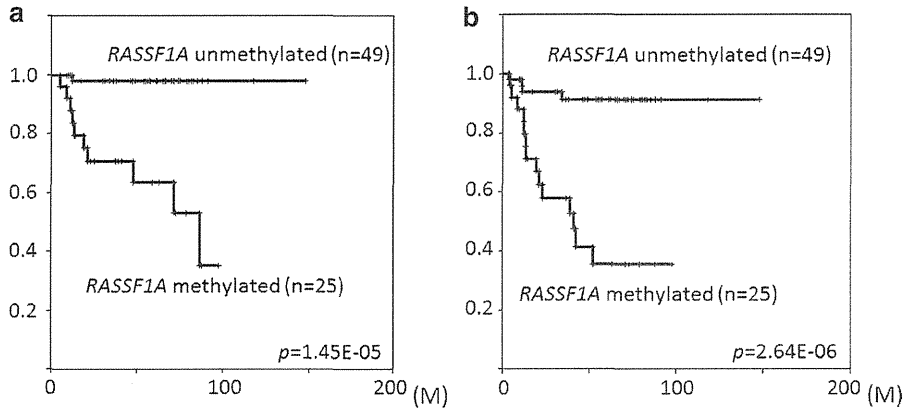

Fig. 3 **a** Overall survival curves and **b** event-free survival curves for hepatoblastoma patients classified by the methylation status of *RASSF1A*

nine tumors in FFPE specimens were classified pathologically into the mixed fetal and embryonal subtype, and DNA was extracted from the tumor cells of each subtype and the normal liver. The other five tumors were the pure fetal subtype, so DNA was extracted from fetal hepatoblastoma and normal liver cells.

The mean methylation values of *RASSF1A* in nine normal liver, nine fetal type, and four embryonal type samples were 8.6, 19.7, and 42.2 %, respectively (Fig. 4). This showed that fetal and embryonal types were significantly associated with *RASSF1A* methylation. Moreover, *RASSF1A* methylation values in samples of the normal liver, fetal type, and embryonal type were elevated in ascending order, when compared to each type of cell taken from the same patient.

Discussion

Complete surgical resection and chemotherapy including cisplatin remains the mainstay of hepatoblastoma treatment. In contrast to standard-risk patients, of who over 90 % achieve long-term survival, the treatment of patients with unrespectable and metastatic disease remains a challenge. Furthermore, there seems to exist a group of patients with high-risk tumors in PRETEXT II, which have a poorer prognosis despite the high-level resectability [5]. First, this study demonstrated that *RASSF1A* methylation was significantly associated with metastatic tumors and a poor prognosis, and that *RASSF1A* methylation may be useful to identify high-risk tumors in PRETEXT II. Secondly, *RASSF1A* methylation was also shown to be histologically correlated with different types of tumor. These findings suggest that *RASSF1A* may be a promising molecular marker to stratify the patients into appropriate risk groups in order to develop better therapeutic approaches.

The present factors predicting the outcome in hepatoblastoma patients include the age at diagnosis, histology, local growth pattern of the tumor, presence of metastasis, and the level of alpha–feto protein [1]. Chromosomal gains of 2q, 8q, and 20, high-level expression of *TERT* or *PLK1*, *CTNNB1* mutation, and *RASSF1A* methylation were shown

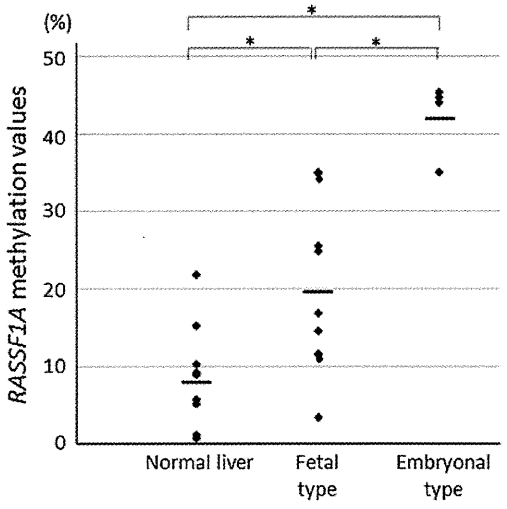

Fig. 4 *RASSF1A* methylation value for each sample is plotted by histological type. *Horizontal bars* indicate the mean value of each type. The p values were calculated by one-way ANOVA followed by Student's t test with Bonferroni correction ($*p < 0.017$)

to be molecular–genetic markers predicting a poor outcome [4, 8, 9]. We have been focusing on *RASSF1A* methylation in hepatoblastomas, since it has been proven to be an independent prognostic factor by multivariate analysis [4]. RASSF1A inhibits tumor formation by apoptosis, and regulates microtubule dynamics and mitotic arrest via multiple effectors. By dysregulation of the Ras-signaling pathway, *RASSF1A* methylation is correlated with poor differentiation and vascular invasion of cancer cells, and an unfavorable outcome [10]. In child cancers, *RASSF1A* methylation was shown to be associated with a poor outcome in neuroblastoma and Wilms tumor [11, 12]. In this study, we newly adopted bisulfite pyrosequencing as a tool for methylation analysis because it is a highly effective and practical method and offers higher throughput compared to quantitative methylation-specific PCR used in the previous study [4]. We believe that bisulfite pyrosequencing can be a reliable tool when used in a clinical setting.

Cairo et al. [13] identified a 16-gene signature discriminating tumors with a fairly well-differentiated histology and a favorable prognosis against advanced and poorly differentiated tumors with a dismal outcome. In this study, *RASSF1A* methylation was also shown to be correlated with different types of histological phenotype by examining FFPE samples dissected separately. As shown in Table 1, there was no apparent difference in *RASSF1A* methylation values between the fetal subtype and the mixed fetal and embryonal subtype, probably because contamination with different types of tumor cell, normal tissues, and mesenchymal components could not be avoided using fresh-frozen specimens. With these gene signatures based on different phenotypes, the molecular classification of hepatoblastoma tumors may become possible after thorough clinical testing. Although the number of cases in this study is too small to draw definite conclusions, we expect that these molecular markers can be used as prognostic markers predicting the treatment outcome when larger clinical trials are carried out.

In conclusion, *RASSF1A* methylation was significantly associated with metastatic tumors and a poor prognosis in hepatoblastoma patients, and it may be especially useful to identify high-risk tumors in PRETEXT II. The *RASSF1A* methylation was also shown to be correlated with different histological phenotypes. We hope that this work will contribute to establishing a useful molecular marker to predict the outcome of hepatoblastoma patients, stratify the patients efficiently, and develop better therapeutic strategies.

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Conflict of interest The authors who have taken part in this study declare that they do not have anything to disclose regarding funding or any conflict of interest with respect to this manuscript. The first and the corresponding authors are JSPS members, and this abstract was selected for presentation at the 50th Annual Meeting of the Japanese Society of Pediatric Surgeons.

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