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Table 4 HR of regression from non-smoking CIN1/2 according to the serum micronutrients and nutrient intake questionnaire

	n	Person-months	Events	Cumulative 2-year rate (95 % CI)	Hazard ratio for regression (95 % CI)			
					Unadjusted	*p* value	Adjusted model	*p* value
Serum retinol							*p* for trend	0.292
Low (<55.2)	62	809.8	39	67.0 (54.5–79.0)	1		1	
Medium (55.2–67.9)	70	922.3	41	62.8 (50.9–74.6)	0.93 (0.60–1.44)	0.75	1.03 (0.65–1.63)	0.908
High (>67.9)	58	743.4	39	71.4 (58.7–83.1)	1.08 (0.69–1.68)	0.742	1.21 (0.74–1.98)	0.448
Serum α-carotene							*p* for trend	0.883
Low (<5.1)	46	560.7	28	64.4 (50.1–78.5)	1.00		1.00	
Medium (5.1–9.7)	62	789.7	38	66.1 (53.3–78.4)	0.97 (0.60–1.59)	0.918	1.22 (0.73–2.05)	0.449
High (>9.7)	82	1,125.1	53	68.7 (57.9–79.0)	0.93 (0.59–1.47)	0.76	1.26 (0.75–2.11)	0.384
Serum β-carotene							*p* for trend	0.206
Low (<28.3)	45	583.9	26	60.1 (45.8–74.7)	1.00		1.00	
Medium (28.3–57.6)	61	780.1	41	75.7 (62.7–86.9)	1.16 (0.71–1.90)	0.557	1.20 (0.71–2.03)	0.488
High (>57.6)	84	1,111.5	52	65.5 (54.8–76.0)	1.03 (0.64–1.65)	0.91	1.23 (0.73–2.07)	0.439
Serum zeaxanthin/lutein							*p* for trend	0.024
Low (<42.9)	56	729.3	34	64.8 (51.4–77.8)	1.00		1.00	
Medium (42.9–57.3)	61	817.3	38	66.7 (54.2–78.9)	1.00 (0.63–1.59)	1	1.12 (0.69–1.84)	0.642
High (>57.3)	73	928.9	47	68.6 (57.1–79.5)	1.05 (0.68–1.64)	0.813	1.25 (0.78–2.01)	0.352
Serum cryptoxanthin							*p* for trend	0.129
Low (<11.2)	47	650.1	28	64.7 (50.0–79.1)	1.00		1.00	
Medium (11.2–22.1)	61	740.7	38	68.2 (55.3–80.4)	1.23 (0.75–2.00)	0.414	1.24 (0.74–2.08)	0.412
High (>22.1)	82	1,084.7	53	67.5 (56.8–77.8)	1.16 (0.73–1.83)	0.536	1.35 (0.82–2.22)	0.231
Serum lycopene							*p* for trend	0.269
Low (<19.8)	63	805.3	37	63.2 (50.7–75.7)	1.00		1.00	
Medium (19.8–35.8)	63	827.7	43	73.8 (61.5–84.8)	1.11 (0.71–1.72)	0.651	1.17 (0.73–1.87)	0.51
High (>35.8)	64	842.5	39	64.3 (52.0–76.4)	1.00 (0.63–1.55)	0.962	1.28 (0.79–2.07)	0.316
Serum α-tocopherol							*p* for trend	0.176
Low (<753.0)	60	731.7	39	67.1 (54.7–79.0)	1.00		1.00	
Medium (753.0–983.9)	63	829.9	40	67.5 (55.2–79.2)	0.91 (0.59–1.42)	0.676	0.96 (0.60–1.53)	0.866
High (>983.9)	67	913.9	40	66.5 (53.9–78.6)	0.81 (0.52–1.26)	0.344	0.96 (0.60–1.54)	0.859
Retinol intake							*p* for trend	0.892
Low (<190.2)	62	760.7	36	63.5 (50.5–76.4)	1.00		1.00	
Medium (190.2–313.1)	63	840.7	41	70.4 (57.9–82.0)	1.04 (0.67–1.63)	0.854	0.90 (0.53–1.54)	0.704
High (>313.1)	65	874.1	42	66.3 (54.5–77.7)	1.02 (0.65–1.59)	0.94	0.86 (0.48–1.53)	0.61
Carotene intake							*p* for trend	0.131
Low (<3,281.4)	47	606.4	29	67.7 (52.7–81.9)	1.00		1.00	
Medium (3,281.4–5,042.8)	71	959.6	40	62.1 (50.0–74.2)	0.88 (0.55–1.43)	0.615	0.89 (0.51–1.56)	0.676
High (>5,042.8)	72	909.5	50	70.8 (59.8–81.0)	1.16 (0.74–1.84)	0.515	1.08 (0.60–1.94)	0.804
Vitamin A intake							*p* for trend	0.134
Low (<2,398.8)	50	676.0	28	63.5 (48.8–78.2)	1.00		1.00	
Medium (2,398.8–3,466.7)	69	934.1	41	63.8 (51.7–75.8)	1.08 (0.67–1.75)	0.755	1.14 (0.65–1.99)	0.654
High (>3,466.7)	71	865.4	50	72.3 (61.3–82.4)	1.42 (0.89–2.25)	0.14	1.47 (0.79–2.73)	0.218
Vitamin E intake							*p* for trend	0.163
Low (<6.7)	51	631.5	29	61.3 (47.4–75.5)	1.00		1.00	
Medium (6.7–8.7)	62	884.3	39	66.0 (53.6–78.1)	0.98 (0.61–1.58)	0.932	1.38 (0.70–2.71)	0.354
High (>8.7)	77	959.7	51	70.3 (59.3–80.6)	1.16 (0.74–1.83)	0.519	1.44 (0.67–3.12)	0.352

Cox's proportional hazard model showing the hazard ratio for regression in a cumulative 24-month period in non-smokers. The adjusted model was identical to the model used in Table 3. The units of micronutrients are expressed as μg/dL

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Table 5 HR of regression from current smoking CIN1/2 according to the serum micronutrients and nutrient intake questionnaire

	n	Person-months	Events	Cumulative 2-year rate (95 % CI)	Hazard ratio for regression (95 % CI)			
					Unadjusted	*p* value	Adjusted model	*p* value
Serum retinol							*p* for trend	0.43
Low (<55.2)	47	614.0	27	64.0 (49.2–78.6)	1		1	
Medium (55.2–67.9)	38	417.6	24	70.5 (53.4–85.7)	1.29 (0.74–2.23)	0.369	1.54 (0.87–2.76)	0.141
High (>67.9)	57	780.5	21	42.9 (30.1–58.3)	0.60 (0.34–1.06)	0.08	0.54 (0.29–1.00)	0.05
Serum α-carotene							*p* for trend	0.898
Low (<5.1)	59	751.9	33	62.5 (49.2–75.8)	1.00		1.00	
Medium (5.1–9.7)	53	689.6	22	49.9 (35.3–66.7)	0.72 (0.42–1.24)	0.24	0.85 (0.48–1.53)	0.595
High (>9.7)	30	370.6	17	61.8 (43.6–80.2)	1.04 (0.58–1.87)	0.886	1.23 (0.63–2.39)	0.537
Serum β-carotene							*p* for trend	0.667
Low (<28.3)	63	788.0	31	58.1 (44.6–72.2)	1.00		1.00	
Medium (28.3–57.6)	53	700.2	27	54.5 (41.1–69.1)	1.02 (0.61–1.71)	0.94	1.07 (0.62–1.86)	0.808
High (>57.6)	26	323.9	14	66.6 (44.5–87.0)	1.06 (0.56–2.00)	0.854	1.04 (0.51–2.14)	0.915
Serum zeaxanthin/lutein							*p* for trend	0.373
Low (<42.9)	54	640.8	32	63.6 (50.0–77.0)	1.00		1.00	
Medium (42.9–57.3)	52	669.4	26	54.1 (40.4–69.0)	0.79 (0.47–1.33)	0.372	0.88 (0.51–1.52)	0.645
High (>57.3)	36	501.9	14	57.6 (37.9–78.8)	0.55 (0.29–1.02)	0.059	0.76 (0.37–1.53)	0.435
Serum cryptoxanthin							*p* for trend	0.866
Low (<11.2)	62	727.3	36	67.4 (53.9–80.2)	1.00		1.00	
Medium (11.2–22.1)	47	644.3	20	48.4 (33.9–65.2)	0.63 (0.36–1.09)	0.098	0.72 (0.39–1.31)	0.279
High (>22.1)	33	440.5	16	53.9 (36.6–73.1)	0.73 (0.40–1.31)	0.286	0.85 (0.44–1.64)	0.63
Serum lycopene							*p* for trend	0.517
Low (<19.8)	43	543.8	21	55.3 (39.9–71.9)	1.00		1.00	
Medium (19.8–35.8)	55	761.7	29	60.8 (46.7–75.1)	0.96 (0.55–1.69)	0.896	0.79 (0.42–1.48)	0.457
High (>35.8)	44	506.6	22	54.4 (39.2–70.9)	1.08 (0.59–1.96)	0.802	0.77 (0.38–1.54)	0.456
Serum α-tocopherol							*p* for trend	0.042
Low (<753.0)	53	594.2	34	68.8 (55.5–81.4)	1.00		1.00	
Medium (753.0–983.9)	49	718.2	19	43.5 (30.1–59.7)	0.47 (0.27–0.83)	0.009	0.53 (0.27–0.94)	0.03
High (>983.9)	40	499.7	19	66.7 (46.0–86.0)	0.64 (0.36–1.11)	0.114	0.76 (0.42–1.40)	0.383
Retinol intake							*p* for trend	0.58
Low (<190.2)	50	573.8	29	62.3 (48.3–76.4)	1.00		1.00	
Medium (190.2–313.1)	51	673.9	25	56.5 (42.1–71.9)	0.74 (0.43–1.26)	0.263	0.76 (0.42–1.37)	0.36
High (>313.1)	41	564.4	18	52.3 (36.2–70.6)	0.63 (0.35–1.13)	0.124	0.57 (0.29–1.13)	0.106
Carotene intake							*p* for trend	0.182
Low (<3,281.4)	64	730.7	34	59.8 (46.9–73.1)	1.00		1.00	
Medium (3,281.4–5,042.8)	43	632.0	22	58.7 (42.7–75.4)	0.72 (0.42–1.24)	0.238	0.71 (0.39–1.31)	0.272
High (>5,042.8)	35	449.4	16	52.9 (35.8–72.2)	0.73 (0.41–1.33)	0.309	0.55 (0.25–1.18)	0.122
Vitamin A intake							*p* for trend	0.268
Low (<2,398.8)	65	723.6	36	61.9 (49.1–74.9)	1.00		1.00	
Medium (2,398.8–3,466.7)	43	642.5	19	49.1 (34.4–66.2)	0.59 (0.34–1.03)	0.064	0.58 (0.31–1.07)	0.081
High (>3,466.7)	34	446.0	17	60.6 (42.2–79.4)	0.74 (0.42–1.32)	0.307	0.60 (0.28–1.32)	0.208
Vitamin E intake							*p* for trend	0.567
Low (<6.7)	61	684.0	32	56.7 (44.1–70.1)	1.00		1.00	
Medium (6.7–8.7)	45	720.6	19	49.0 (34.4–66.0)	0.56 (0.32–0.99)	0.047	0.51 (0.25–1.05)	0.066
High (>8.7)	36	407.5	21	67.3 (49.6–83.8)	1.02 (0.59–1.77)	0.947	0.56 (0.23–1.38)	0.211

Cox's proportional hazard model showing the hazard ratio for regression in a cumulative 24-month period in current smokers only. The adjusted model was identical to the model used in Table 3. The units of micronutrients are expressed as μg/dL

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Table 6 HR of progression from entire CIN1/2 according to the serum micronutrients and nutrient intake questionnaire

	n	Person-months	Events	Cumulative 5-year rate (95 % CI)	Hazard ratio for progression (95 % CI)			
					Unadjusted	*p* value	Adjusted model	*p* value
Serum retinol							*p* for trend	0.372
Low (<55.2)	128	4,588.2	7	8.7 (3.6–20.1)	1.00		1.00	
Medium (55.2–67.9)	132	5,048.8	17	17.1 (10.8–26.6)	2.25 (0.93–5.44)	0.071	2.35 (0.95–5.77)	0.063
High (>67.9)	131	5,210.1	14	14.3 (8.5–23.7)	1.82 (0.73–4.51)	0.198	2.23 (0.88–5.60)	0.089
Serum α-carotene							*p* for trend	0.669
Low (<5.1)	127	4,506.6	13	15.4 (8.7–26.2)	1.00		1.00	
Medium (5.1–9.7)	133	4,955.5	17	16.0 (10.0–25.0)	1.21 (0.59–2.49)	0.609	1.08 (0.51–2.31)	0.835
High (>9.7)	131	5,385.0	8	9.6 (4.7–19.0)	0.52 (0.22–1.27)	0.153	0.46 (0.18–1.15)	0.098
Serum β-carotene							*p* for trend	0.337
Low (<28.3)	129	4,245.0	18	21.8 (13.6–33.9)	1.00		1.00	
Medium (28.3–57.6)	131	5,208.1	7	7.0 (3.2–14.7)	0.32 (0.13–0.77)	0.011	0.28 (0.11–0.71)	0.007
High (>57.6)	131	5,394.0	13	13.2 (7.7–22.3)	0.58 (0.28–1.19)	0.14	0.52 (0.24–1.13)	0.098
Serum zeaxanthin/lutein							*p* for trend	0.772
Low (<42.9)	130	4,611.4	11	12.1 (6.7–21.4)	1.00		1.00	
Medium (42.9–57.3)	130	5,291.5	17	17.9 (11.2–28.0)	1.37 (0.64–2.94)	0.415	1.58 (0.71–3.53)	0.266
High (>57.3)	131	4,944.2	10	9.4 (5.1–17.1)	0.87 (0.37–2.06)	0.756	0.95 (0.39–2.32)	0.908
Serum cryptoxanthin							*p* for trend	0.618
Low (<11.2)	129	4,591.6	12	12.2 (6.9–20.9)	1.00		1.00	
Medium (11.2–22.1)	130	4,906.2	16	17.1 (10.6–27.0)	1.26 (0.60–2.67)	0.544	1.37 (0.61–3.06)	0.445
High (>22.1)	132	5,349.3	10	10.5 (5.5–19.7)	0.73 (0.32–1.69)	0.465	0.71 (0.29–1.72)	0.450
Serum lycopene							*p* for trend	0.286
Low (<19.8)	129	4,827.0	15	17.5 (10.5–28.3)	1.00		1.00	
Medium (19.8–35.8)	131	4,954.6	11	10.0 (5.6–17.6)	0.71 (0.33–1.55)	0.395	0.61 (0.27–1.36)	0.223
High (>35.8)	131	5,065.5	12	13.1 (7.3–22.9)	0.76 (0.36–1.63)	0.48	0.73 (0.33–1.59)	0.428
Serum α-tocopherol							*p* for trend	0.788
Low (<753.0)	128	5,143.1	11	12.0 (6.6–21.2)	1.00		1.00	
Medium (753.0–983.9)	132	5,052.6	11	13.3 (7.4–23.3)	1.01 (0.44–2.33)	0.983	0.91 (0.39–2.10)	0.820
High (>983.9)	131	4,651.4	16	15.7 (9.3–25.8)	1.60 (0.74–3.45)	0.232	1.87 (0.84–4.19)	0.126
Retinol intake							*p* for trend	0.666
Low (<190.2)	130	4,778.5	14	14.7 (8.6–24.4)	1.00		1.00	
Medium (190.2–313.1)	130	4,985.2	15	16.7 (9.8–27.7)	1.02 (0.49–2.12)	0.948	1.08 (0.51–2.32)	0.834
High (>313.1)	131	5,083.4	9	9.5 (4.9–17.7)	0.60 (0.26–1.40)	0.239	0.62 (0.23–1.68)	0.346
Carotene intake							*p* for trend	0.331
Low (<3,281.4)	130	4,578.9	9	10.8 (5.2–21.6)	1.00		1.00	
Medium (3,281.4–5,042.8)	131	4,789.0	16	17.6 (11.4–26.7)	2.02 (0.91–4.46)	0.083	2.30 (0.97–5.42)	0.058
High (>5,042.8)	130	5,479.2	10	11.6 (6.2–21.0)	0.94 (0.38–2.33)	0.901	1.19 (0.41–3.44)	0.746
Vitamin A intake							*p* for trend	0.493
Low (<2,398.8)	130	4,510.5	11	12.2 (6.3–22.9)	1.00		1.00	
Medium (2,398.8–3,466.7)	131	4,921.0	16	15.1 (9.4–23.9)	1.33 (0.62–2.87)	0.463	1.32 (0.59–2.97)	0.500
High (>3,466.7)	130	5,415.6	11	12.6 (3.8–22.2)	0.84 (0.36–1.95)	0.689	0.92 (0.33–2.54)	0.873
Vitamin E intake							*p* for trend	0.834
Low (<6.7)	130	4,431.0	12	13.8 (7.5–24.7)	1.00		1.00	
Medium (6.7–8.7)	130	5,128.1	15	14.1 (8.6–22.6)	1.08 (0.51–2.31)	0.842	1.06 (0.44–2.56)	0.892
High (>8.7)	131	5,288.0	11	12.5 (6.8–22.1)	0.78 (0.34–1.77)	0.55	1.00 (0.30–3.38)	0.998

Cox's proportional hazard model showing the hazard ratio for progression over a cumulative 60-month period. The adjusted model was identical to the model used in Table 3. The units of micronutrients are expressed as μg/dL

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effects were weaker or not found with a higher level of serum beta-carotene (HR 0.52, 95 % CI 0.24–1.13, $p = 0.098$). In contrast, a high carotene intake did not show an inverse relationship, but rather a non-significant increase in progression (HR 2.30, 95 % CI 0.97–5.42, $p = 0.058$). There was no significant association between other serum micronutrients and risk for CIN progression.

Discussion

The role of environmental factors, including micronutrients and tobacco smoking, in cervical carcinogenesis has been discussed. Smoking status in particular interfered with serum levels and intake of carotenoids as shown in Tables 1 and 2. In smokers, food intake is intrinsically lower than in non-smokers [22]. From the questionnaires, the intake per day of all micronutrients, except retinol and tocopherol, was lower in current smokers than in non-smokers, suggesting an unbalanced diet resulting from either smoking or other lifestyle behaviors (Table 1). Serum levels of alpha-carotene, beta-carotene and cryptoxanthin were inversely correlated with smoking status, but alpha-tocopherol was not correlated with smoking status after adjusting for age, BMI and frequency of alcohol intake (Table 2). These data were consistent with a previous report in which smoking was shown to affect serum beta-carotene levels but to have no effect on alpha-tocopherol levels [23]. Though alpha-tocopherol and beta-carotene are well known as antioxidants, the antioxidant effect of alpha-tocopherol is not due to a reaction with oxygen. In contrast, beta-carotene does react with oxygen. This suggests that there is a difference in the mechanisms of antioxidant reaction [24].

In regression subjects, we expected to find a protective effect from high serum levels or intake of carotenoids; however, neither of these had protective effects. We assume that smoking status modulates dietary intake or serum levels of micronutrients. Therefore, we investigated the association between dietary intake or serum levels of micronutrients and CIN regression, taking into account smoking status (Tables 3, 4, 5). In non-smoking regression subjects, regression was significantly related to the serum levels of zeaxanthin/lutein. This relationship was not found in current smokers. In a similar example, an isoflavone has a protective effect for lung cancer, but the effect is abolished by smoking [25]. It was reported that zeaxanthin/lutein may be a useful marker of intake of leafy vegetables, spinach, green peas, broccoli and seaweed [26]. Zeaxanthin/lutein is chemically more hydrophilic than other carotenoids such as alpha- and beta-carotene, lycopene and beta-cryptoxanthin. The mechanisms of a potential protection against carcinogenesis may include: induction of apoptosis, inhibition of angiogenesis, enhancement of gap junction intercellular communication, induction of cell differentiation, prevention of oxidative damage, and modulation of the immune system. Serum levels of lutein have been inversely associated with cytochrome CYP1A2 activity, a hepatic enzyme responsible for the metabolic activity of a number of putative human carcinogens [27]. High serum levels of alpha-tocopherol tend to have an inhibitory effect on regression in smokers (Table 4). There is a similar effect in that supplemental vitamin E, presumably causing a high concentration of alpha-tocopherol, is associated with an increased risk of lung cancer, which was confined to current smokers [28]. Alpha-tocopherol is considered to be an antioxidant, but it might act as a pro-oxidant [24].

Though a weak and non-significant protective effect of dietary intake or low serum concentration of beta-carotene has been observed previously [10, 15, 29, 30], we found that a medium serum level of beta-carotene showed a significant protective effect on CIN progression, whereas this protective effect at higher serum levels of beta-carotene was weaker or abolished (Table 6). These data appear to be consistent with in-vitro experiments reporting that very high concentrations of beta-carotene decreased antioxidant and/or induced pro-oxidant effects [31, 32]. Based on epidemiological studies that have shown an association between a low intake of carotenes and human cancers [33], an intervention study was conducted for the prevention of lung cancer [34]. However, it was paradoxically reported that high serum levels of beta-carotene induced by oral supplements promoted lung cancer in male heavy smokers aged 50–69 years. In CIN, oral beta-carotene supplementation did not enhance CIN regression in a randomized, double-blind phase III trial [35]. One explanation for these failures may be that oral supplements induced extremely high serum levels of beta-carotene. Taken together, these data suggest that medium serum levels of beta-carotene may interfere with CIN progression or cancer development.

There was a discrepancy between the results of dietary intake and serum levels of beta-carotene. Endogenous metabolic processes may influence the serum concentrations of micronutrients. In fact, inconsistent results of the serum levels and dietary intake of alpha-tocopherol in patients with prostate cancer, and contradictory results of retinol in patients with cervical cancer, have been reported previously [14, 36, 37]. Additionally, there is limited dietary intake information obtained from questionnaires because of inherent recall bias. We examined the residual confounding factors, including passive smoking, the number of sexual partners, and serum *Chlamydia* IgG antibody, in addition to the adjusted model. Despite confounding by other risk factors included for adjustments, the analyses did not change the conclusion.

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To our knowledge, this is the first large-scale prospective cohort study for CIN outcome to report an association between serum levels of antioxidant micronutrients adjusted for potential confounders including CIN grade, HPV genotype, age, total energy intake and smoking. To make our comparisons, we investigated not only serum levels but also dietary intake of micronutrients, despite the fact that food-intake questionnaires contain limited information. It is known that the accuracy of recalling past dietary intake is influenced by current dietary habits [38]. There are inconsistent results between previous case–control and cohort studies. However, our discrepant results did not reach the conclusion that women with CIN received a benefit from consuming a beta-carotene-rich diet. However, not smoking and maintaining high serum levels of zeaxanthin/lutein, presumably by intake of leafy vegetables, spinach, green peas, broccoli, and seaweed, are advantageous for the prevention of cervical cancer.

This study has some potential limitations. We included only CIN patients with an available serum sample for measurement of serum nutrients [18]. The majority of CIN patients already had persistent HPV infection at enrollment in the present study. If these nutrients play an important role in preventing persistent HPV infection, we cannot determine that role in this cohort study. The food intake contains not only the micronutrients being investigated but also other nutrients and mixtures. The incident number of progression cases was small and it was difficult to analyze by smoking status. A large-scale cohort study with a longer period of observation is required to clarify the association between serum levels or dietary intake of micronutrients and the risk of developing cervical cancer.

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Conflict of interest The authors declare that they have no conflict of interest.

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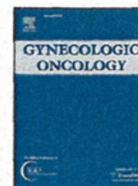

Effective treatment of pelvic lymphocele by lymphaticovenular anastomosis

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HIGHLIGHTS

- Pelvic lymphocele is a major complication after pelvic lymphadenectomy.
- We performed lymphaticovenular anastomosis (LVA) on pelvic lymphoceles, and found that LVA was highly effective regardless of the lymphoceles' size.
- LVA could be considered as an initial treatment for lymphoceles.

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ABSTRACT

Objective. Pelvic lymphocele can be a severe complication associated with surgical procedures such as pelvic lymphadenectomy. Lymphaticovenular anastomosis (LVA) is increasing in popularity as a surgical treatment for lymphedema. The aim of this study was to evaluate whether LVA is an effective treatment for lymphocele, which is caused by an obstruction of the lymphatic flow in a manner similar to the development of lymphedema.

Methods. Eleven female patients, who presented with lymphocele, were treated with LVA. Before the operation, 3 of them were treated with a percutaneous catheter. Lymphocele size and the volume of daily drainage were measured before and after LVA.

Results. The lymphocele was completely resolved in 6 patients and partially resolved in the remaining 5 patients. The mean size of the pelvic lymphocele changed from 400 ml (range 50–1050 ml) to 43 ml (range 0–120 ml) ($P<0.01$). In the 3 patients who had percutaneous drainage catheters, the volume of fluid drained decreased from 340 ml/day to 20 ml/day after LVA.

Conclusions. Our technique is minimally invasive and is performed under local anesthesia. LVA is effective regardless of the size of the lymphocele. Therefore, LVA should be considered as a therapy for lymphocele because of its low invasiveness and its effectiveness in re-establishing circulation of lymphatic flow. Further studies should be performed to compare LVA with other minimally invasive techniques, such as percutaneous catheter and sclerotherapy.

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Introduction

A lymphocele is defined as an abnormal collection of lymph fluid, without an epithelial lining, at the site of lymphatic surgery [1,2]. A pelvic lymphocele can occur after surgical procedures such as pelvic lymphadenectomy for gynecologic or prostatic malignancies and renal transplantation [2–7] and has an incidence of 1–49% [1,2,8]. Most lymphoceles are small and asymptomatic, and they disappear spontaneously with time. However, when sufficiently large, they may lead to symptoms such as abdominal pain, infection, increased urinary frequency, hydronephrosis, deep venous thrombosis, and lower extremity lymphedema [1,2,6,8].

Several treatment options are available for the management of pelvic lymphoceles; however, there is no consensus as to which is most effective. Needle aspiration and percutaneous catheter drainage, which are commonly used in the initial management of symptomatic lymphoceles, have reported initial cure rates of up to 80%, but treated lymphoceles are often complicated by infection (in up to 50% of cases) and recur in 80–90% of cases [1,2,9]. The cure rate of sclerotherapy is also reported to be between 77% and 98%, but the success of this treatment is inversely proportional to the size of the lymphocele—larger lymphoceles are more likely to be symptomatic and cause complications; thus, the effectiveness of this therapy is limited [1,9]. Laparoscopic or surgical fenestration is the most invasive of the current therapies

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for pelvic lymphoceles and is often reserved for refractory cases. Complications associated with this approach include perforation of the bladder, transection of the ureter, and injury of pelvic vessels [6,10].

Lymphaticovenular anastomosis (LVA) using supermicrosurgery has been reported as being a simple, minimally invasive, and effective treatment for secondary lymphedema of the upper and lower extremities [11–15]. This technique bypasses proximal lymphatic blockages, providing an alternative route for lymphatic fluid recirculation into the venous system. We reasoned that a similar principle could be used to treat pelvic lymphoceles. By providing an alternative route of lymphatic drainage into the venous system for lymphatic fluid from the lower limb, the flow of lymphatic fluid into the lymphocele would be reduced. Furthermore, we have previously demonstrated that valvular incompetence permits a reversal of the lymphatic flow in cases of lymphedema, and we hypothesized that a similar mechanism in the postsurgical pelvic lymphatic system of patients with lymphoceles would allow drainage of the lymphocele through the newly created LVAs. We have previously reported successful management of a pelvic lymphocele using this approach in a single patient [16]. In this study, we report our experience using this technique to treat pelvic lymphoceles in a series of 11 patients.

Materials and methods

Patients

Eleven female patients with pelvic lymphoceles were referred to our department between May 2010 and October 2011. All the patients had undergone treatment for gynecologic cancer (see Table 1). The presence of a pelvic lymphocele was determined in all cases by a CT scan. In 3 patients, a percutaneous catheter had been inserted prior to referral in an attempt to treat the lymphocele, but drainage had remained unacceptably high.

Preoperative preparation

All patients gave fully informed consent for the procedure, acknowledging that current outcome data on efficacy was unknown. One day before each operation, fluorescence lymphatic imaging, using a near-infrared fluorescence imaging device (Photodynamic Eye, Hamamatsu Photonics, Hamamatsu City, Japan), was performed after the injection of indocyanine green dye (ICG) to identify the lymphatic channels in both lower limbs, as previously described [17–19]. The location of the lymphatic channels was marked, facilitating the accurate placement of short incisions and thereby allowing the procedure to be performed under local anesthesia. In those patients with a percutaneous drainage device in situ, the drain was clamped after lymphatic mapping in order to increase pressure in the lower-limb lymphatics and facilitate LVA.

Operative technique

Under local anesthesia, 2 or 3 incisions (2 cm each) were made on each lower limb—on the dorsum of the foot, the distal medial thigh, and the groin—overlying previously mapped lymphatic channels [20]. Dissection of superficial lymphatic channels and venules was performed under magnification using the operative microscope. Lymphaticovenular anastomosis was performed using either 11/0 or 12/0 nylon sutures in an end-to-end (Fig. 1A and B) or side-to-end configuration. The patency of anastomoses was confirmed by either washout of the venous lumen by lymphatic flow or venous backflow into the lymphatic channels. Wounds were closed with intradermal 4/0 PDS and interrupted 5/0 nylon sutures.

Postoperative management

Twice daily for 7 days after surgery, 60 μg of prostaglandin E1 (Prostandin; Ono Pharma. Co., Osaka, Japan) was injected intravenously. Prostaglandin is used for dilation of the vessels and seems to result in decreased occlusion of the anastomosis site. Compression therapy was started on postoperative day 14. All but one patient had follow-up CT scans.

Assessment

Assessment of the lymphocele was performed by either CT or ultrasonography. The volume of the lymphocele was calculated as an ellipsoid. Statistical analysis of the data was performed using a Wilcoxon test. A P value less than 0.05 was deemed significant.

Results

The demographic details of the patients and the details of their gynecologic treatment are shown in Table 1. We performed a mean of 8.2 lymphaticovenular anastomoses, with a mean venule diameter of 0.70 mm and a mean lymphatic diameter of 0.55 mm. In 6 of the 11 patients, the pelvic lymphocele was completely resolved after LVA, and in the remaining 5 patients, the lymphocele was partially resolved. The average pelvic lymphocele size was 400 ml (range 50–1050 ml) on preoperative CT scan and 43 ml (range 0–120 ml) on postoperative CT ($P<0.01$). In the 3 patients who underwent preoperative placement of percutaneous drainage catheters, the mean volume of fluid drained each day was reduced from 340 ml to 20 ml after LVA (Fig. 2).

Prior to our operations, 10 patients had symptoms: 1 had hydronephrosis requiring a urinary stent, 1 had frequent pre-ileus, 2 had increased urinary frequency, 2 had infection of lymphoceles, 3 had abdominal pain, and 7 had lower-extremity lymphedema. All symptoms except for lymphedema were alleviated after the LVA operation, and the lymphedema was improved from the pre-LVA state. No patients in

Table 1
Patient data.

Age	Site of primary cancer	Stage	Primary operation	Pre-LVA lymphocele volume [ml]	Post-LVA lymphocele volume [ml]	Catheter inserted
52	Cervical cancer	Ib	RH, BSO, PLA	60	0	−
63	Cervical cancer	IVb	RH, SILA, PALA	160	0	+
42	Endometrial cancer	Ic	RH, BSO, PLA	50	0	−
53	Endometrial cancer	Ib	RH, BSO, PLA	1050	0	+
53	Endometrial cancer	Ic	TAH, BSO, PLA	700	0	−
66	Endometrial cancer	Ic	RH, BSO, PLA, PALA	200	110	−
42	Ovarian cancer	Ic	TAH, BSO, PLA, PALA,	460	50	−
53	Ovarian cancer	Ia	TAH, BSO, PLA, PALA, pOM	170	90	−
56	Ovarian cancer	IIIc	Secondary EILA	500	100	+
61	Ovarian cancer	Ic	TAH, BSO, PLA, PALA, pOM	700	0	−
69	Ovarian cancer	Ic	TAH, BSO, PLA, PALA, pOM	350	120	−

RH: radical hysterectomy, BSO: bilateral salpingo-oophorectomy, PLA: pelvic lymphadenectomy, SILA: superficial inguinal lymphadenectomy, TAH: abdominal total hysterectomy, pOM: partial omentectomy, EILA: external iliac lymphadenectomy, PALA: para-aortic lymphadenectomy.

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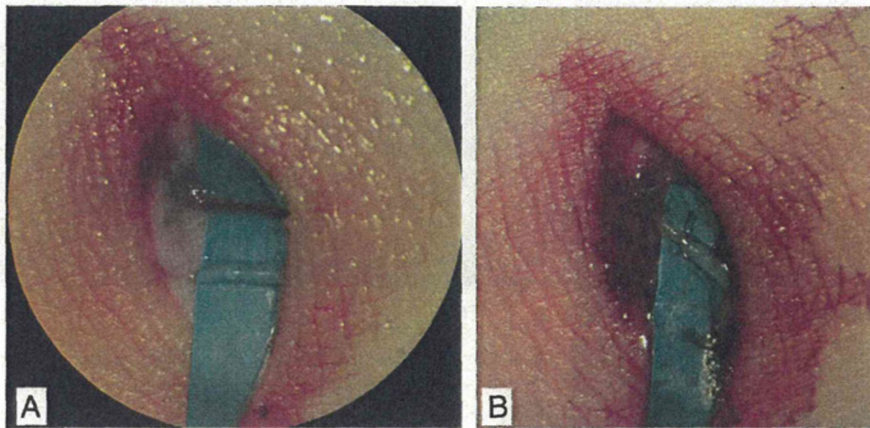

Fig. 1. Lymphaticovenular anastomosis. (A) Pre-anastomosis image. The upper vessel is a vein; the lower image is a lymphatic channel. (B) Post-anastomosis image. The anastomosis was performed with 5 sutures of 11–0 nylon. The left side of the anastomosis looks clear because the lymphatic fluid is under higher pressure than venous blood; therefore, flow from the lymphatic channel on the right washes out blood from inside the vein.

our series suffered any complications of LVA. Specifically, there were no infections and no wound-healing problems.

Representative case

The lymphocele was detected on CT 11 days before LVA (Fig. 3A). The patient had abdominal pain, urinary frequency, and lower-extremity lymphedema. A percutaneous catheter was inserted 3 days before the operation and the daily volume of drained fluid was recorded until the tube was removed (Fig. 3D). The operation site of LVA was noted (Fig. 3B). A CT image taken 3 days after the operation showed that the lymphocele had disappeared (Fig. 3C). The catheter was removed after confirming that the symptoms had disappeared.

Discussion

Since Teruel et al. [21] first reported successful sclerotherapy with povidone iodine for lymphoceles, several types of sclerotherapy with a variety of agents have been reported [1,6,9]. The cure rate for sclerotherapy is reported to be between 77% and 98% [1], and the recurrence rate is 31% [22]. However, the success of this treatment is inversely proportional to the size of the lymphocele [1]—larger lymphoceles are more likely to be symptomatic and cause complications; thus, when the lymphoceles most require treatment, this therapy is likely to be relatively less effective.

Laparoscopic or open surgical fenestration can be used to open a pathway from the lymphocele into the peritoneal cavity, allowing the peritoneum to absorb lymphatic fluid [23]. These techniques enable lymphatic fluid to re-circulate into the venous system. However, they are more invasive than other therapies and have been associated with complications including bladder perforation, ureter transection, and injury of pelvic vessels [6,10]. Recurrence can occur with closure of the fenestrated window in 6–15% of cases [6,24].

The ideal therapy for lymphoceles would be more effective and less invasive than traditional treatment methods (including sclerotherapy and surgical fenestration), with fewer complications and a lower chance of recurrence. Moreover, restoration of lymphatic circulation, broken by lymphadenectomy, is desired.

LVA is emerging as the treatment of choice for lymphedema of the extremities. Before the LVA operation was available, only conservative therapies, such as massage and compression garments, could be used for lymphedema. These techniques do not enable re-establishment of lymphatic fluid circulation into the venous system, but simply release it into the trunk lesion. Therefore, patients are never able to discontinue the therapy if they wish to reduce the edematous lesion. LVA was

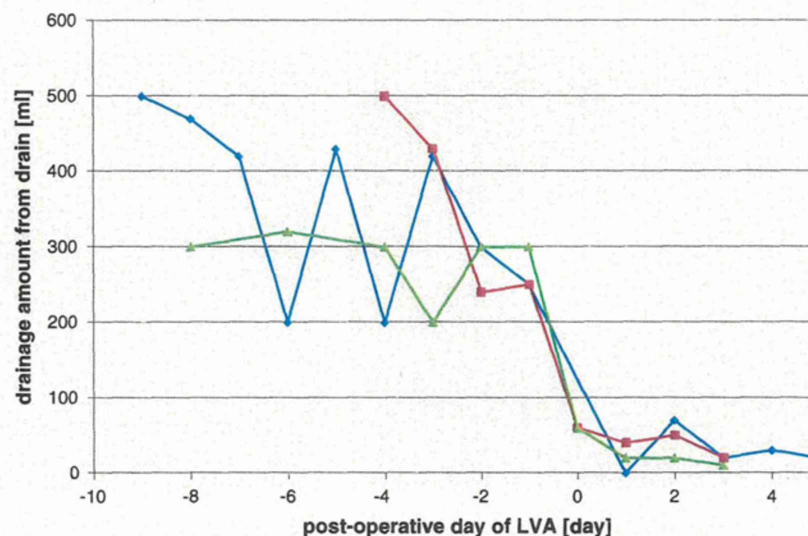

Fig. 2. Daily drainage from 3 patients who had preoperative placement of percutaneous drains. Note the dramatic decrease in drainage following LVA.

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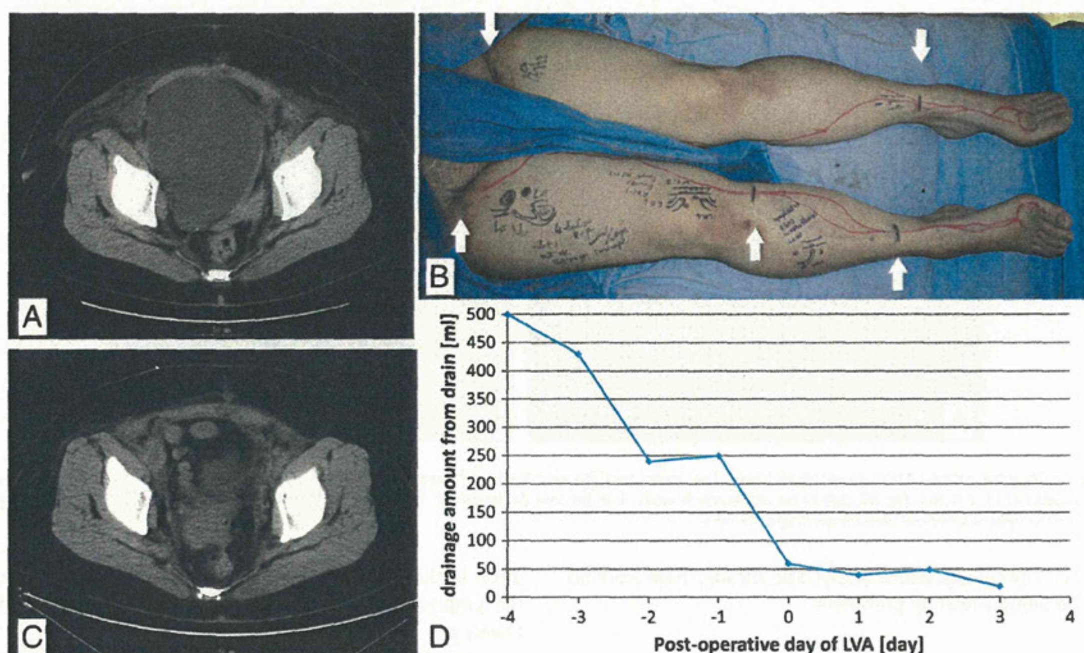

Fig. 3. Resolution of pelvic lymphocele after LVA. (A) CT scan of a large pelvic lymphocele after gynecologic surgery. (B) Immediate postoperative view. Seven anastomoses were performed through five 3-cm incisions under local anesthesia. (C) A CT scan of the same patient 3 days after LVA demonstrates complete resolution of the lymphocele. (D) The drainage chart of the same patient demonstrates large daily drainage volumes before LVA. Following LVA, the volume of fluid drained was dramatically reduced, and the drain was removed on postoperative day 4.

introduced as a new concept for lymphedema therapy [13–15]. The aim is to bypass proximal lymphatic blockages that cause congestion of lymphatic flow and thereby provide an alternative route for lymphatic fluid recirculation. Although the lymphatic channels normally have autokinetic movement because of smooth muscles, when the muscle damage due to lymphedema is irreversible, compression therapy is needed as an adjuvant therapy to direct lymphatic fluid into venulae. However, when the damage is dormant, the muscles react by pushing lymphatic fluid into the venous system. In this case, the patients do not need to receive any further adjuvant therapy.

In lymphoceles, the lymphatic flow from the lower limbs is similarly interrupted at the surgical region, where it flows into the cavity. This is illustrated in Fig. 4, where ICG injected into the dorsum of the feet is seen to escape into the percutaneous drainage catheter of a lymphocele. We reasoned that LVA would enable the lymphatic flow from the limbs to bypass the lymphocele, reducing its volume and preventing lymphatic flow into the lymphocele, thereby allowing spontaneous resolution. Our results supported this hypothesis, with total recovery in 6 of the 11 cases and improvement in the remaining 5 cases.

We believe that LVA has multiple advantages over the other methods currently used to treat lymphoceles. First, LVA is minimally invasive because it can be performed under local anesthesia and requires only 2 or 3 small skin incisions. Second, LVA is effective for

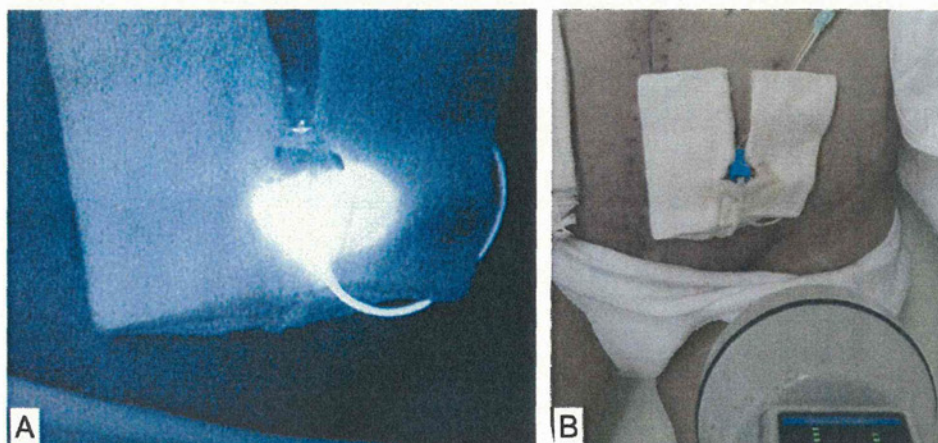

Fig. 4. Demonstration of lymphatic flow from the leg into a pelvic lymphocele. (A) Fluorescence lymphatic image of a percutaneous catheter, 5 min after injecting ICG into the first dorsal web space of the foot, indicates the lymphatic flow from the leg rapidly entering the lymphocele. It also indicates that the fluorescing root is the dominant lymphatic channel pouring into the lymphocele. (B) Conventional photograph of the same area. The Photodynamic Eye camera used to obtain the picture in (A) is seen at the bottom of the picture.

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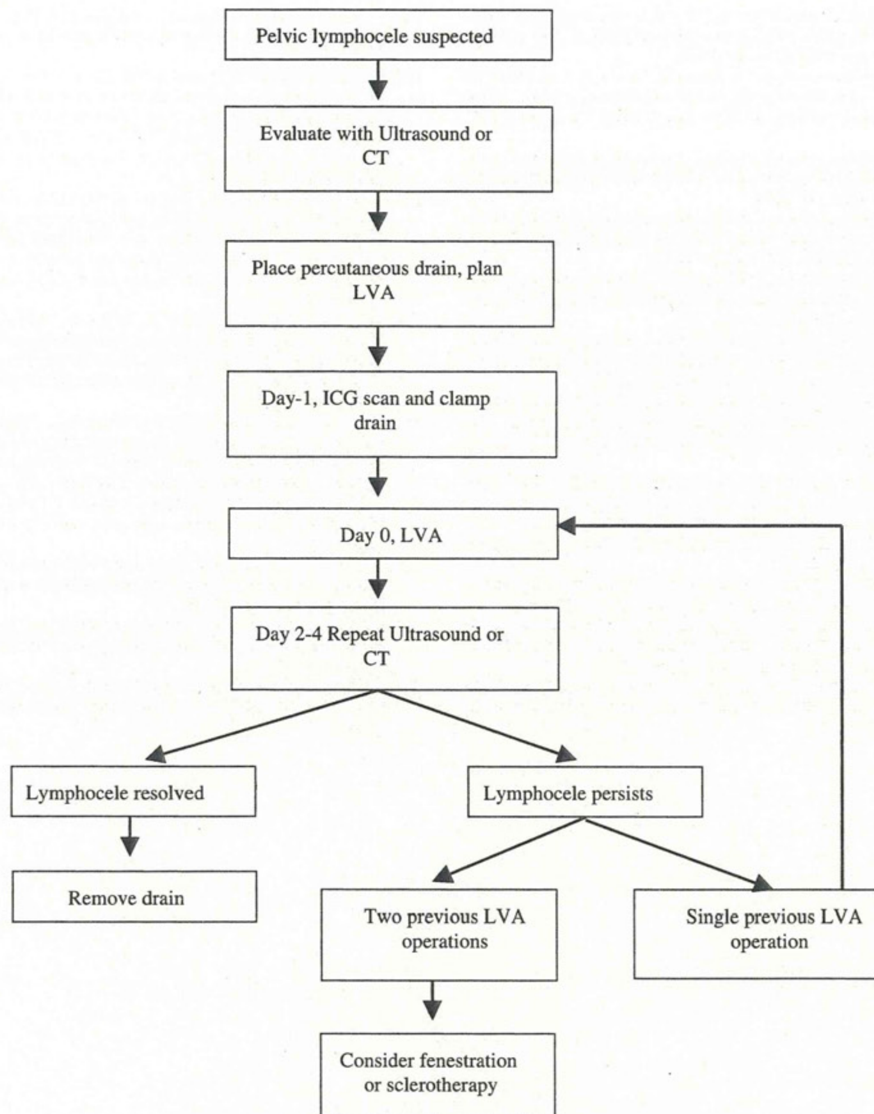

Fig. 5. Algorithm for the management of pelvic lymphocele.

all sizes of lymphocele. Third, LVA can prevent or improve lymphedema, which is a common complication of pelvic lymphadenectomy. This is in contrast to other techniques that resolve the fluid collection by blocking or sclerosing lymphatic channels, which may in itself provoke the development of lymphedema.

Our experience of reverse lymphatic flow with the valvular incompetence of lymphatic channels in lymphedema indicates that lymphatic flow into the lymphocele from places other than the leg may occur in a retrograde pattern into the leg's lymphatic channels and then into the venous system. Competent lymphatic valves may account for the partial failure of our technique, and we recommend that a percutaneous catheter be used to drain the remaining fluid if it is symptomatic.

Unfortunately, LVA is not perfectly effective for all patients. We suggest that the reason for this is that the lymphatic channels used for LVA are sometimes not the dominant lymphatic channels for the lymphoceles. In such cases, the lymphocele could diminish but not vanish. A second LVA might be able to locate the dominant lymphatic channel. Other therapies could also be used: LVA is an indirect approach to the lymphocele whereas other therapies approach lymphoceles directly. We present our algorithm for management of pelvic lymphoceles in Fig. 5.

In conclusion, our technique is minimally invasive and is performed under local anesthesia. It is therefore suitable for patients who have recently undergone major pelvic surgery. LVA should be considered as an initial therapy for lymphoceles because of its low invasiveness, high effectiveness, and ability to re-establish circulation of lymphatic flow. Further studies should be performed to compare LVA with other minimally invasive techniques, such as percutaneous catheter and sclerotherapy.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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Regulation of SIRT1 determines initial step of endometrial receptivity by controlling E-cadherin expression

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ABSTRACT

Sirtuin 1 (SIRT1), originally found as a class III histone deacetylase, is a principal modulator of pathways downstream of calorie restriction, and the activation of SIRT1 ameliorates glucose homeostasis and insulin sensitivity. We examined the role of SIRT1 in the regulation of uterine receptivity using Ishikawa and RL95-2 endometrial carcinoma cell lines. Exogenous expression of SIRT1 significantly enhanced E-cadherin expression, while small interfering RNA-mediated depletion of endogenous SIRT1 resulted in a significant reduction of E-cadherin expression. A SIRT1 activator resveratrol elevated E-cadherin expression in a dose dependent manner, while SIRT1 repressors nicotinamide and sirtinol exhibited a dose dependent reduction of E-cadherin expression. We also showed that both forced expression of SIRT1 and activation of SIRT1 promote E-cadherin-driven reporter gene constructs, and SIRT1 is localized at E-cadherin promoter containing E-box elements in Ishikawa cells. Using an *in vitro* model of embryo implantation, we demonstrate that exogenous expression of SIRT1 and stimulation of SIRT1 activity resulted in the Ishikawa cell line becoming receptive to JAR cell spheroid attachment. Furthermore, resveratrol enhanced E-cadherin and Glycodelin protein expression at sites of intercellular contact, suggesting an additive role of resveratrol in promoting implantation. The initial step of human reproduction depends on the capacity of an embryo to attach and implant into the endometrial wall, and these results revealed the novel mechanism that activation and increased expression of SIRT1 play an important role in uterine receptivity.

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1. Introduction

After the blastocyst attaches onto the endometrial glandular epithelium, broad adhesion, transient disruption of the uterine epithelium by degradation of extracellular matrix, and penetration into the uterine stroma occurs. The implantation window is hormonally regulated and is characterized by changes in the molecules expressed by uterine epithelial cells [1]. Although numerous cell surface components including adhesion molecules, cytokines, growth factors, and lipids are postulated to be involved in implantation, only a few genes are essential to this process [2,3]. The study of implantation is both technically and ethically difficult to investigate *in vivo*. *In vitro* study using primary tissues possesses many limitations due to the individual variations. Currently endometrial epithelial carcinoma cell lines such as Ishikawa [4] and RL95-2 [5] cells, and trophoblast cell lines have been used to mimic the receptive state of the uterine epithelium in *in vitro* implantation assays, and the *in vitro* implantation assay is regarded as a useful model for studying mechanisms of human implantation [4–6].

E-cadherin would be involved in the initial attachment of embryos because E-cadherin is found on luminal epithelium and on trophectoderm [7]. E-cadherin in uterine endometrium was known to be hormonally regulated because E-cadherin expression is significantly enriched at the apical membranes of mouse uterine epithelial cells during the preimplantation stage [8]. Embryos lacking functional E-cadherin by targeted disruption exhibit defective preimplantation development and failure to implant [9]. E-cadherin is known to maintain organized architecture and plays a pivotal role in the regulation of epithelial cell proliferation, differentiation, and survival [10]. In addition, genetic or epigenetic alterations of E-cadherin expression have been often associated with various cancers and a class III histone deactylase (HDAC), SIRT1, is linked to the E-cadherin expression [11,12].

Abbreviations: AICAR, 5-aminoimidazole-4-carboxyamide ribonucleoside; AMPK, AMP-activated kinase; HDAC, histone deacetylase; NAM, nicotinamide.

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SIRT1, the mammalian homologue of yeast Sir2 (silent information regulator 2), deacetylates multiple targets in mammalian cells [13]. By regulating various molecules, SIRT1 functions as a master regulator of energy homeostasis, transcriptional regulation, heterochromatin formation, genomic stability, p53 function, and cell survival [14]. SIRT1 is associated with the oncogenic functions because SIRT1 promotes cell survival by inhibiting acetylated-p53 dependent apoptosis [15,16]. SIRT1 has been shown to be involved in the maintenance of gene silencing by associating with CpG island of promoter regions in tumor suppressor genes [11]. However, SIRT1 also possesses anti-oncogenic function because SIRT1 inhibits Survivin expression by changing the epigenetic modification of histone H3, and a phytochemical compound resveratrol (trans-3,5,40-trihydroxystilbene) mimic the inhibitory effects of SIRT1, thus serves as an anti-carcinogenic compound [17]. Polyphenols have been known to activate SIRT1 either directly or indirectly, and the deacetylating activity of SIRT1 can be inhibited by nicotinamide (NAM) [13]. Resveratrol is an indirect activator of SIRT1 and has been shown to activate the expression of nicotinamide phosphoribosyltransferase and AMP-activated kinase (AMPK) [18–20]. In addition, SIRT1 and AMPK mutually affect the functions of each other [18,21]. So far, it is not known whether these chemicals are able to modulate the expression of E-cadherin.

To better understand the functional significance and the transcriptional regulation by SIRT1, we studied the effect of the transcriptional regulation of E-cadherin driven by SIRT1. We demonstrate that E-cadherin expression is regulated by SIRT1 in endometrial carcinoma cells. Either activation of SIRT1 or increased expression of SIRT1 plays a key role in the development of human uterine receptivity via inducing E-cadherin expression. These findings establish a principal biological function of SIRT1 in the modulation of E-cadherin function, and further identify SIRT1 as a possible determinant and potential therapeutic target in implantation failure.

2. Materials and methods

2.1. Cell culture and chemicals

Ishikawa human endometrial adenocarcinoma cell line was kindly provided by Dr. M. Nishida (Tsukuba University, Ibaraki, Japan). RL95-2 cells (CRL-1671, human endometrial adenocarcinoma), 293T cells (CRL-11268, human embryonic kidney cells), and JAR cells (HTB-144, human choriocarcinoma cells) were purchased from the American Type Culture Collection (Manassas, VA, USA). Resveratrol, NAM, and sirtinol were from Sigma–Aldrich (St. Louis, MO, USA). AICAR (5-Aminoimidazole-4-carboxyamide ribonucleoside) was purchased from Cell Signaling Technology (Danvers, MA, USA).

2.2. Western blot

To determine the effect of SIRT1 functions, cells were treated with indicated concentrations of resveratrol, sirtinol, NAM, or AICAR. Western blot analysis and immunostaining were performed as described previously [22].

2.3. RNAi

The ablation of SIRT1 and DBC1 was performed by transfection of the Ishikawa cells and RL95-2 cells with small interfering RNA (siRNA) duplex oligos synthesized by Invitrogen (Carlsbad, CA, USA) and Qiagen (Hilden, Germany). Control siRNA (AllStars Negative Control siRNA, 1027281) and DBC1-specific siRNA [DBC1-RNAi: 5′ AAACGGAGCCUACUGAACA 3′, which covered mRNA regions of nucleotides 1379–1397 (amino acids 460–466) of DBC1, and KIAA1967-RNAi, SI00461853] were transfected by using HyperFect reagent (Qiagen). Stealth RNAi Duplex (Invitrogen) specific for SIRT1 (Oligo ID: HSS118729, HSS177403 and HSS117404) was transfected by using Lipofectamine RNAi MAX (Invitrogen).

2.4. Luciferase assay

Transfection was performed with Effectene reagent (Qiagen) according to the manufacturer's recommendation. For luciferase assay, indicated expression vectors were cotransfected with E-cad(−108)-Luc or E-cad(−108)Mut-Luc [23]. As an internal control to equalize transfection efficiency, phRL CMV-*Renilla* vector (Promega Corp., Madison, WI, USA) was also transfected in all the experiments. Individual transfections, each consisting of triplicate wells, were repeated at least three times [22].

2.5. In vitro implantation assay

To generate spheroids of JAR cells for use as blastocyst models, the JAR cells were grown in suspension in petri dishes at a density of 2×10^5 cells/ml. Petri dishes were placed on a slow shaker in 37 °C, 5% CO_2 humidified incubator overnight. During this incubation, JAR cells form spheroids of 50–200 μm in diameter through natural aggregation. Ishikawa cells were seeded in quintuplicate in 24-well plates and incubated until they reached subconfluent monolayers. Thereafter, Ishikawa cells were treated with indicated chemical compounds. On the day of the assay, co-culture of Ishikawa cells and JAR spheroids (approximately 100 spheroids/well) for 24 h was performed. After incubation, the monolayers were gently washed twice with PBS to remove unattached spheroids. Cell monolayers were then examined under light microscope for spheroids numbers.

2.6. Fluorescence microscopy

Ishikawa cells were grown on 12 mm BD BioCoat (BD Biosciences, Franklin Lakes, NJ, USA) glass coverslips in 6-well plates. Spheroids of JAR cells seeded on the Ishikawa monolayer cells were fixed with PBS containing 4% paraformaldehyde. After blocking, Ishikawa cell and spheroids were incubated sequentially with anti-E-cadherin (610181, BD Biosciences) and anti-Glycodelin (EP870Y, Novus Biologicals, CO, USA) antibodies. Secondary antibodies were Alexa fluor 488 conjugated donkey anti-mouse IgG (A-21428), and Alexa fluor 555 conjugated goat anti-rabbit IgG (A-21202, Invitrogen). The slides were briefly counter-stained and analyzed under the confocal fluorescence microscope (Carl-Zeiss Micro Imaging Inc., Oberkochen, Germany).

2.7. Chromatin immunoprecipitation assay

Preparation of soluble Ishikawa chromatin for PCR amplification was performed essentially as described [24]. Primers to amplify E-cadherin promoter region containing three E-box domains are as follows; forward, 5′-GTGAACCCTCAGCCAATCAG-3′; reverse, 5′-TCACAGGTGCTTTGCAGTTC-3′.

3. Results

3.1. E-cadherin expression is regulated by SIRT1 deacetylase

To determine the effect on E-cadherin expression, nonpolar Ishikawa and RL95-2 cells were transfected with siRNA oligos. In contrast to the previous study using breast cancer cell line MDA-MB-231 [11] and prostate cancer cell line PC3 and DU145 [12],

RNAi-mediated knockdown of SIRT1 expression resulted in a decreased expression of E-cadherin in endometrial cancer cell lines (Fig. 1A, C). Thus our data demonstrate that SIRT1 has a critical role in regulating the expression level of E-cadherin in endometrial cancer cells. The forced expression of SIRT1 revealed that the increased expression of SIRT1 resulted in an increased expression of E-cadherin (Fig. 1B, D), confirming that E-cadherin expression was paralleled by the SIRT1 expression. DBC1 is a negative regulator of SIRT1 deacetylase function [15,16], and we investigated the possibility that the regulation of E-cadherin expression by SIRT1 is affected by DBC1. However, DBC1 expression remained unchanged by siRNA-mediated knockdown of SIRT1. The result that SIRT1 is able to stimulate E-cadherin expression led us to examine the role of SIRT1 in the activation of E-cadherin promoter. Transient transfection assays were performed using an E-box-wild type luciferase [E-cad(−108)-Luc] or E-box-mutated [E-cad(−108)Mut-Luc] reporter plasmid, carrying minimum promoter region (−108 to + 125 bp) for the E-cadherin expression. The E-box elements (consensus sequence 5'-CANNTG-3') were originally shown to be binding regions of zinc-finger transcription factors such as SLUG and SNAIL that repressed E-cadherin-driven reporter gene constructs and three E-box sites within the promoter have been demonstrated to drive the expression of E-cadherin [23]. Although SIRT1 efficiently elevated the promoter activity of the reporter plasmid in 293T cells in a dose dependent manner, the transactivation function of SIRT1 was not observed by the expression of E-cad(−108)Mut–Luc in luciferase assays (Fig. 1E). To test whether SIRT1 is indeed recruited to E-cadherin promoter, we performed a chromatin immunoprecipitation assay using the E-cadherin gene promoter containing three E-box elements. As expected, clear recruitment of endogenous SIRT1 to the target sequence in the E-cadherin promoter was observed (Fig. 1F). Thus our results suggest that SIRT1 plays a significant role in the E-cadherin expression, and E-box domains were shown to be crucial for the SIRT1-mediated expression of E-cadherin.

3.2. Stimulation of SIRT1 function increases the expression level of SIRT1 and E-cadherin

We next hypothesized that expression of E-cadherin would be regulated by small molecules that govern SIRT1 function. In concordance with the previous report, SIRT1 expression was also stimulated by resveratrol (Fig. 2A, B). Contrary to this, Ishikawa cells exposed to sirtinol (Fig. 2C) and NAM (Fig. 2D) exhibited decreased expression of E-cadherin in a dose-dependent manner. AICAR is an AMPK activator and AMPK enhances SIRT1 activity by increasing cellular NAD^+ levels [21]. We also showed that AICAR stimulated E-cadherin and SIRT1 expression in Ishikawa and RL95-2 cells, but the extent of increase by AICAR was relatively modest compared to that by resveratrol (Fig. 2E). We further examined the ligand-induced transactivation function of E-cadherin promoter, and the resveratrol-induced transactivation was dose-dependent with a roughly estimated concentration value required for one-half maximal activation (EC_{50}) of about 10 μM (Fig. 2F).

3.3. SIRT1 expression and activity affect the spheroid attachment to the Ishikawa cell monolayer

We next examined whether the expression of SIRT1 and its activity affected the implantation capacity of Ishikawa cells using an *in vitro* model of embryo attachment. Forced expression of SIRT1 in Ishikawa cells exhibited a 1.3-fold increased number

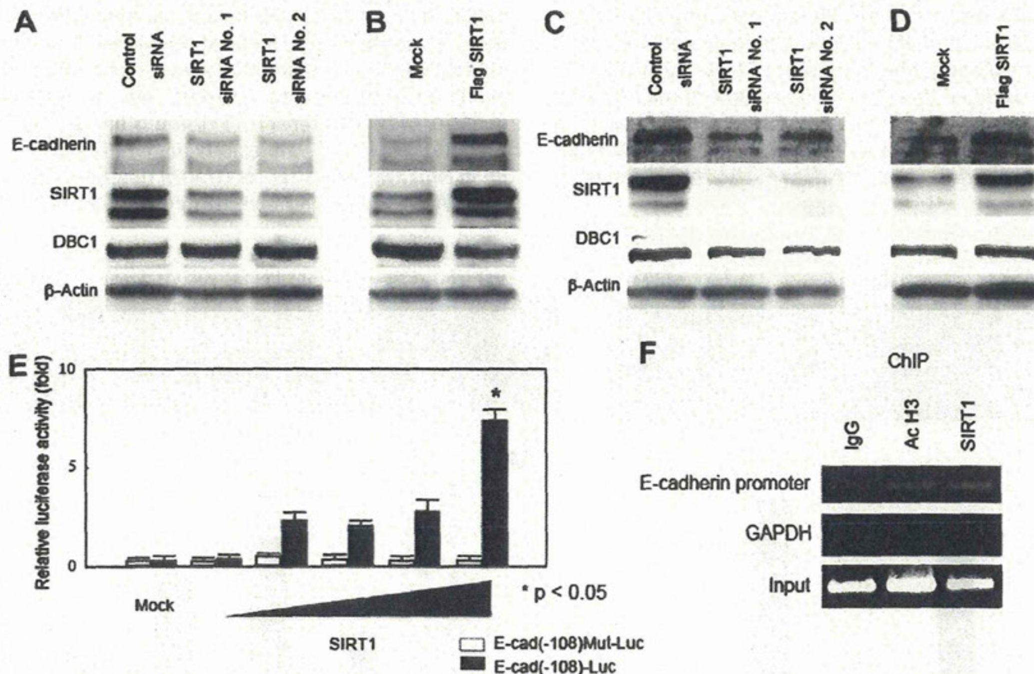

Fig. 1. E-cadherin promoter dependent regulation of E-cadherin expression by SIRT1 deacetylase. Ishikawa (A, B) and RL95-2 (C and D) cells were transfected with expression vector of SIRT1 or indicated siRNA and analyzed by Western blotting. The immunoblotting analysis using anti-SIRT1 antibodies revealed that the expression of SIRT1 paralleled the expression of E-cadherin in cell lysates. (E) 293T cells were transfected with the indicated amounts of SIRT1 expression plasmids (pcDNA Flag SIRT1), and transfected whole cell lysates were assayed for luciferase activity produced from the reporter plasmid [E-cad(−108)-Luc or E-cad(−108) Mut-Luc]. SIRT1 showed a dose-dependent stimulation of the transactivation function of E-cadherin promoter, while E-cadherin promoter possessing mutations within three E-box domains failed to show activation function of SIRT1. (F) Chromatin immunoprecipitation assay was performed to confirm the recruitment of SIRT1 at E-cadherin gene promoter, a region containing three E-box domains. AcH3 denotes acetylated histone H3.

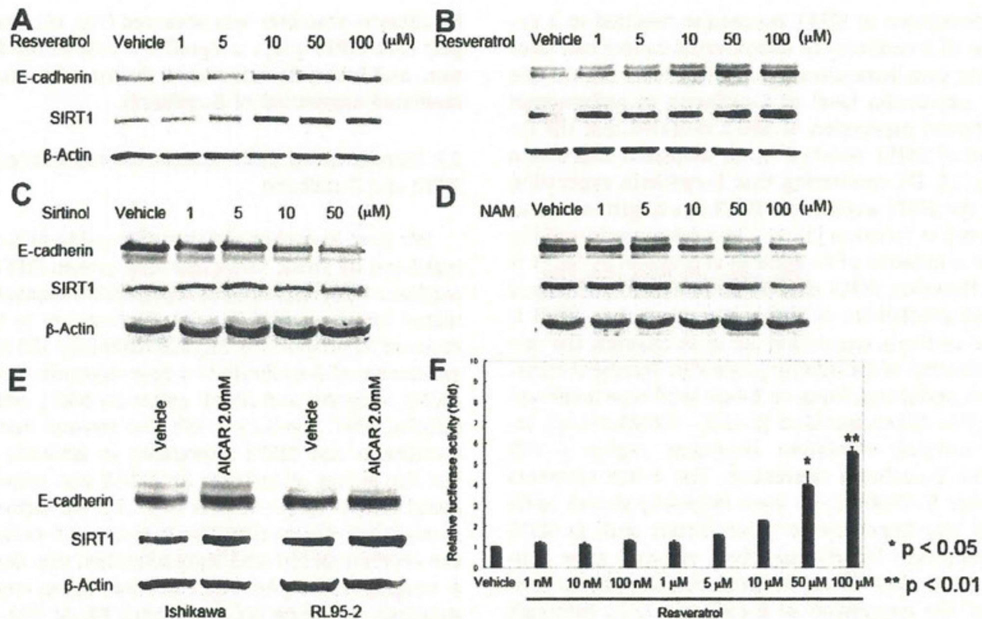

Fig. 2. Activation of SIRT1 using chemical compounds resulted in an increased expression of E-cadherin and SIRT1. Ishikawa (A, C, D, and E left panel) and RL95-2 (B, E right panel) cells were treated with various doses of vehicle, SIRT1 activators (resveratrol and AICAR), and SIRT1 repressors (sirtinol and NAM). Twenty-four hours after treatment, cells were harvested and protein expression of E-cadherin and SIRT1 was analyzed by Western blot. The immunoblotting analysis revealed that SIRT1 activators increased the expression of E-cadherin and SIRT1 in cell lysates, while SIRT1 repressors decreased the expression of E-cadherin and SIRT1 in cell lysates. (F) 293T cells were transfected with E-cad(−108)-Luc plasmids, and treated with various doses of resveratrol. Resveratrol showed a dose-dependent transactivation function of E-cadherin promoter.

of spheroids attached to the cell monolayers, while siRNA-mediated depletion of endogenous SIRT1 resulted in a 4-fold decreased uterine receptivity (Fig. 3B). Then we tested whether activation or inactivation of SIRT1 may modulate the uterine receptivity. Ishikawa cells treated with resveratrol exhibited a 3.5-fold greater number of spheroids attached to cell monolayers compared with cells treated with vehicle alone, while repression of SIRT1 by sirtinol resulted in a 2-fold decrease in spheroid attachment to the Ishikawa cell monolayer (Fig. 3D). Thus, the expression of SIRT1 and its activity influenced on the initial attachment of embryos through the mechanism involved in enhanced human uterine receptivity.

3.4. Localization of E-cadherin and Glycodelin to sites of intercellular contact is essential for initial attachment

To further pursue the role of resveratrol, other implantation molecules such as Glycodelin and Survivin were examined. Glycodelin is a progesterone-induced glycoprotein secreted into uterine luminal cavity by endometrial glands in secretory phase [4]. Survivin is an anti-apoptotic molecule and is overexpressed in the majority of human cancers [17], and is upregulated in early gestation [25]. We found significantly higher protein levels for Glycodelin in Ishikawa cells after resveratrol treatment but not for Survivin (Fig. 4A, B). We further investigated whether resveratrol possesses

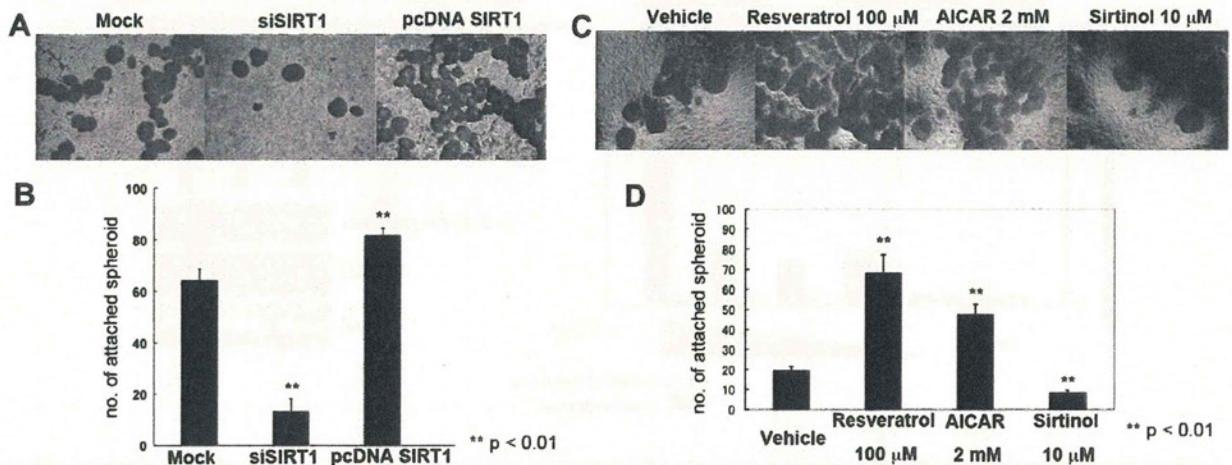

Fig. 3. SIRT1 expression and activity affects uterine receptivity. Spheroid adhesion to Ishikawa cell monolayer was examined by *in vivo* spheroid attachment assay. (A) Representative phase-contrast micrographs of JAR spheroids attached to Ishikawa cell monolayer. (B) Graph showing spheroid adhesion to Ishikawa cell monolayer. Appropriate controls were also analyzed (scramble siRNA and empty pcDNA vector), and no significant difference was observed. (C) Representative phase-contrast micrographs of JAR spheroids attached to Ishikawa cell monolayer. (D) Graph showing spheroid adhesion to Ishikawa cell monolayer after the treatment with chemical compounds.

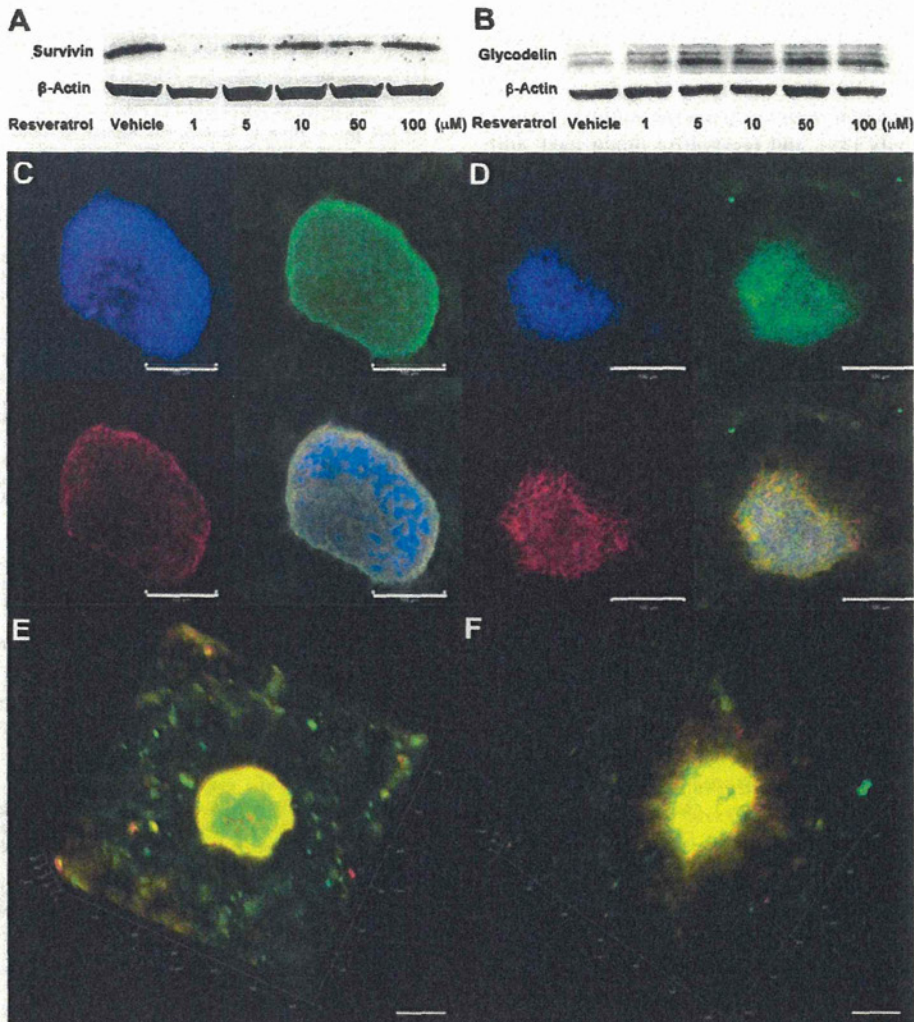

Fig. 4. Expression of implantation-related proteins and colocalization of E-cadherin and Glycodelin in Ishikawa cells. (A, B) Ishikawa cells were treated with various doses of resveratrol and protein expression of Survivin and Glycodelin was analyzed by Western blot. (C–F) In *in vivo* spheroid assay, colocalization of E-cadherin and Glycodelin was examined by immunofluorescence study. (C and D) spheroids attached to the cell monolayer at 1 h (C) and at 24 h (D) are shown. (E, F) Three dimension views of attached spheroids at 1 h (E) and at 24 h (F) are shown. Note that the colocalization signal (yellow; merge) is becoming intense. Bars indicate 100 μm. Results shown are representative photographs.

an additional effect on the uterine receptivity compared with SIRT1 alone. Immunofluoresence study was performed to localize E-cadherin and Glycodelin expression at sites of intercellular contact under the confocal microscopy. Although E-cadherin and Glycodelin were expressed at sites of intercellular contact, colocalization signal was not significant in spheroid cells after 1 h incubation (Fig. 4C). However, both E-cadherin and Glycodelin are intensively expressed and the degree of colocalization signal was significantly elevated in spheroid cells showing dispersing morphological changes after 24 h incubation (Fig. 4D), suggesting that E-cadherin stimulates initial step of spheroid attachment and Glycodelin helps to invade spheroids in endometrial lining. Since both SIRT1 expression and SIRT1 stimulation by chemical compounds increase expression of E-cadherin and Glycodelin, these data confirmed that SIRT1 plays pivotal roles in initial step of implantation.

4. Discussion

The studies of SIRT1 in uterine physiology are limited. A recent study examined the SIRT1 and SIRT2 expression and regulation in human intrauterine tissues [26]. In the present study, we report the possibility that SIRT1 expression and SIRT1 function can regulate human implantation because the stimulation of E-cadherin expression and small molecules that affect SIRT1 activities produced a substantial effect on spheroid attachment ability in an *in vitro* model of endometrial receptivity in nonpolar endometrial cancer cells. Our data was different from the previous observations that SIRT1 is involved in epigenetic silencing of DNA-hypermethylated tumor suppressor genes in breast cancer cells [11], and that SIRT1 serves as a positive regulator of epithelial-mesenchymal transition and metastatic growth of prostate cancer cells [12]. Further investigation should be required to determine the mode of regulation of E-cadherin expression whether this difference could be simply attributed to the difference of cell line.

It is also interesting that resveratrol treatment resulted in an increased expression of SIRT1 protein levels. Our previous study using rat ovarian granulosa cells also demonstrated resveratrol treatment was associated with an increased expression of SIRT1 [14]. The enhancement of SIRT1 expression by resveratrol was considerably potent compared to that by AICAR. This was consistent with the report that the enhancement of SIRT1 expression by AICAR is at best

1.3-fold [27]. Our data provided a novel anti-tumorigenic property of SIRT1 activating chemicals because decreased E-cadherin expression has been identified in a wide variety of malignancies including endometrial cancer [28]. While the underlying mechanism remains elusive, it has been shown that resveratrol has the ability to activate SIRT1 deacetylase activity [29], and resveratrol might have anti-tumorigenic properties. It was reported that resveratrol inhibited the proliferation of a wide variety of human cancer cell lines through the induction of S-phase cell cycle arrest and apoptosis [30] while presenting very low cytotoxicity in animal models. Interestingly, low doses of resveratrol can sensitize cells to low doses of cytotoxic anti-cancer drugs, therefore resveratrol is expected to facilitate the efficacy of anticancer therapy in various human cancers [31].

Suberoylanilide hydroxamic acid (vorinostat), a HDAC inhibitor drug utilized as an anti-cancer drug, has been shown to induce differentiation of endometrial glandular cells and to increase the expression of Glycodelin [4]. Glycodelin was postulated to be secreted from cell that exhibited interactions between spheroids and endometrial lining (Ishikawa cells) because Glycodelin was not found in cultured media from Ishikawa cells [32]. We believe that both Glycodelin protein expression and E-cadherin protein expression synergistically help to improve initial steps of implantation, including attachment, adhesion, and invasion. Considering the teratogenicity of vorinostat in the treatment of infertility, our result is fascinating and may provide a possibility for the practical use of resveratrol because adverse events associated with resveratrol intake were gastrointestinal symptoms alone [33]. The study revealed that repeated administration of high doses of resveratrol resulted in micromolar plasma concentrations. Therefore, our data that elevated E-cadherin expression was observed at a dose of micromolar concentrations of resveratrol can be tolerable and accomplishing dose.

In conclusion, our data indicate that SIRT1 plays an important role in regulating E-cadherin expression. Therefore, SIRT1-activating chemicals including resveratrol and AICAR would be novel therapeutic targets for improvement of initial step of implantation, thereby improvement of assisted reproductive technology success can be expected. However, in view of the difference in the pathophysiology of implantation between cultured cells (*in vitro*) and human (*in vivo*), our data should be interpreted with caution and the present observations should be further verified.

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Genome-Wide Single Nucleotide Polymorphism Arrays as a Diagnostic Tool in Patients With Synchronous Endometrial and Ovarian Cancer

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Objective: Synchronous carcinomas in the endometrium and ovaries can be a single primary tumor with metastasis (SPM) or dual primary tumors (DP). Although the prognosis of DP without any metastases is significantly better than that of SPM, pathological diagnosis is difficult in tumors with similar histological features.

Materials and Methods: In 10 tumors from 5 patients with synchronous endometrial and ovarian carcinomas, 250K single nucleotide polymorphism arrays were performed. The patients were genetically diagnosed according to the pattern of copy number alterations (CNAs), in addition to microsatellite status and mutational analysis of *PIK3CA*, *PTEN*, *K-Ras*, and *CTNNB1*.

Results: Of the 5 patients, 3 exhibited identical CNA patterns, including type, loci, and degree of each alteration in the endometrial and ovarian carcinomas. The other 2 exhibited CNAs only in either endometrial or ovarian carcinoma. All 5 tumors had 1 or more genetic mutations in the genes examined. One patient exhibited mutations both in *PIK3CA* and *PTEN* at discordant sites between endometrial and ovarian carcinomas, whereas the other 4 exhibited concordant mutations. Overall, 4 of the 5 patients were genetically diagnosed with SPM, and the remaining 1 with DP. The pathological diagnosis was not in agreement with the genetic diagnosis in 4 of the 5 patients.

Conclusions: Genome-wide genotyping diagnosis may represent a useful approach for distinguishing between SPM and DP in synchronous endometrial and ovarian carcinomas.

Key Words: Synchronous carcinomas, Endometrial cancer, Ovarian cancer, SNP arrays, Genetic diagnosis

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