表1 次世代HPVワクチンの開発状況

ワクチンの種類	キャリア	開発の進行状況	開発者
予防ワクチン			
1）9価ワクチン	L1-VLP	Ph-III 追跡中	MSD（メルク）
2）L2ワクチン	キメラ VLP	前臨床	Kanda ら，Kleinschmidt ら，Chackerian ら
	Multimeric L2	前臨床	Roden ら
治療ワクチン			
1）全身免疫誘導	表2参照	Ph-I/IIb まで	表2参照
2）粘膜免疫誘導	乳酸菌	Ph-I/IIa 実施中	筆者ら

HPV16/18のどちらかのHPV-DNAもしくはHPV抗体が陽性の集団（既感染者）では，HPV16/18によるCIN2+の予防効果はプラセボ群と比較して有意な差はなかった[8,9]．高い力価の抗体を誘導しても，すでに感染しているHPVによるがん化を阻止できるわけではないということを意味する．このような状況から現行HPVワクチンだけでは子宮頸がんは撲滅できない．これらの問題点を補うことができる次世代HPVワクチンの開発が進められている．現時点で実用化に近いところまで研究・開発が進んでいる次世代HPVワクチンは，①予防ワクチンとして「全タイプのHPV感染を予防するbroad-spectrumワクチン（L2ワクチン，9価ワクチン）」，②治療ワクチンとして「CINに対する治療薬としての治療ワクチン」が挙げられる（**表1**）．

L2ワクチン（予防ワクチン）の開発

現行のHPVワクチンで用いられているL1-VLPがタイプ特異的な感染予防効果を示すことは，すでに筆者らを含め多くの研究で明らかにされていた[10]．国立感染症研究所（当時）の神田忠仁博士は，全タイプの感染をブロードに予防できる次世代のブロードHPVワクチンの開発を続けている．筆者も神田博士のもとその開発に携わってきた．

筆者らは，現行のHPVワクチンで用いられているL1蛋白質ではなく，もう1つの粒子蛋白質であるL2に注目した．筆者らは，HPV16型L2の56-135アミノ酸（aa）が粒子表面に露出していることを確認した．しかもこの領域のアミノ酸配列は，全タイプのHPVに高度に保存されていた[11]（**図1**）．近年ではL2蛋白質の中で最適のブロードHPVワクチンの候補領域として，HPV16型のL2，11-88aa付近が注目されている[12,13]．L2を利用した次世代の全タイプ型予防ワクチンは，実現すれば大変有望な手段となる．

現時点でのL2ワクチンの課題は抗原性が低いことである．L2については抗L2抗体がどの程度ヒトで誘導されるかが未知であり，non-responderがどの程度の割合になるかを確かめなければならない．このL2ワクチンの弱点を補うべくさまざまなワクチン

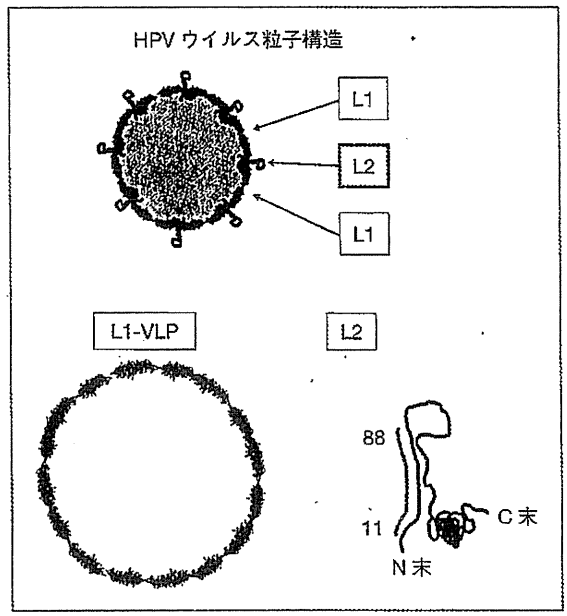

図1　ウイルス粒子構造と L1 と L2

抗原の選定が世界中で進んでいる[14, 15]．例えば，L1-VLP の一部を L2 の中和領域に置換したキメラ VLP や，L2 の中和領域を 8 つの繰り返し構造にした精製蛋白質など抗原性を高めるための工夫が開発されている．これらの工夫によって，マウスやウサギでは良好な抗体誘導能が証明されているが，ヒトにおいて抗体誘導能が得られるかは今度実施されるであろう臨床試験の結果を待たなければ何ともいえない状況である．L2 ワクチンが次世代 HPV 予防ワクチンとして実用化できるかどうかはこの点にかかっているといえる．

9 価 HPV ワクチン（予防ワクチン）の開発

4 価ワクチンを開発した MSD 社は，次世代 HPV（予防）ワクチンとして broad-spectrum に HPV 感染を予防できる 9 価 HPV ワクチン（開発コード No.：V503）を開発している[16]．このワクチンは，6，11，16，18 型に加えて 31，33，45，52，58 型の感染を予防できると期待されている．ワクチン抗原は 4 価ワクチンと同じ L1-VLP であるが，このワクチンは 9 つの型の L1-VLP をカクテルにしている．5 タイプの HPV を加えたことにより，子宮頸がんから検出される HPV の 87％をカバーしていることになる．9 つの HPV タイプに対する感染予防効果が十分に得られれば，子宮頸がんの大部分は 9 価ワクチンによって予防できることになる．2007 年 9 月から V503 の第Ⅲ相臨床試験が米国を中心に始まっている．約 15,000 人を登録し，コントロールアームとしてガーダシル® 接種を置き，主要効果として各タイプによる子宮，膣，外陰疾患の発生頻度と各タイプの抗体価を置き，副次効果として各タイプの持続感染と抗体

表2 HPV治療ワクチンの臨床試験

試験	標的分子	ワクチンキャリア	接種法	対象疾患	開発機関
Ph-I/II	L1, E7	キメラVLP	皮下注	CIN2～3	NCI
Ph-II	E7	Hsp融合蛋白質	皮下注	CIN2～3	Stressgen社
Ph-I/II	E6, E7	ワクチニアウイルス	皮下注	頸がん	Xenova社
Ph-II	L2, E6, E7	L2E6E7融合蛋白質	筋注	CIN2～3	Xenova社
Ph-IIb	E6, E7	プラスミドDNA	筋注	CIN2～3	Zycos社
Ph-IIb	E7	ワクチニアウイルス	筋注	CIN2～3	Roche社
Ph-I	E6, E7	プラスミドDNA	筋注	CIN2～3	VGX社
Ph-I/IIa	E7	*Lactobacillus casai*	経口	CIN3	筆者

陽転率としている．2011年2月現在すでに登録は終了しており，現在追跡中である．

霊長類を用いた動物実験では9価ワクチン接種によって各タイプに対する中和抗体が，いずれも4価ワクチンと同等レベル（疾患予防効果が期待できるレベル）で誘導されるという（第26回国際パピローマウイルス学会，2010，カナダ）．ヒトにおける抗体誘導能のデータは現在行われている臨床試験の結果を待たなければならないが，ヒトでも同様の結果が得られれば，broad-spectrum予防ワクチンとして有望である．近い将来，9価HPVワクチンが実用化されることと期待している．

HPV治療ワクチンの開発の経緯

CIN患者の多くは免疫応答，特に細胞性免疫応答によって，CIN病変を制御している．このことはHIV患者やステロイド常用者のような細胞性免疫が不全状態にある患者でCIN病変が進行しやすいことに基づく考え方である．患者の抗HPVの細胞性免疫応答には個体差があり，CINが進行するか否かを決定する因子になりうることが示されている[17]．治療的ワクチンは，この抗HPV細胞性免疫能を強制的に獲得させ，CIN病変を制御するものである．CINに対する細胞性免疫の標的はE7がん蛋白質が有力である．E6，E7が子宮頸がんのがん形質の維持には重要であるが，免疫療法の標的分子としては，E7がより魅力的である．というのはE7のほうがヒトにおける抗原性が高いからである．たとえば抗E6抗体が検出されるという報告はないが，抗E7抗体の検出については子宮頸がん患者血清をはじめ多くの報告がある．また発現量や細胞内局在もE7のほうがより強いと考えられる[18]．これまでのHPV治療ワクチンの臨床試験のほぼすべてがE7を標的としている．

このように免疫療法の標的としてHPV分子は大変魅力的である．実際，**表2**に示すようにこれまでに多くの臨床試験が海外で実施されてきた．にもかかわらずこれまでにHPV治療ワクチンが実用化されていない理由は，子宮頸部局所での抗HPV細胞性免疫誘導能が問題であると考えられる．**表2**でわかるように，これまでの試験は，皮下

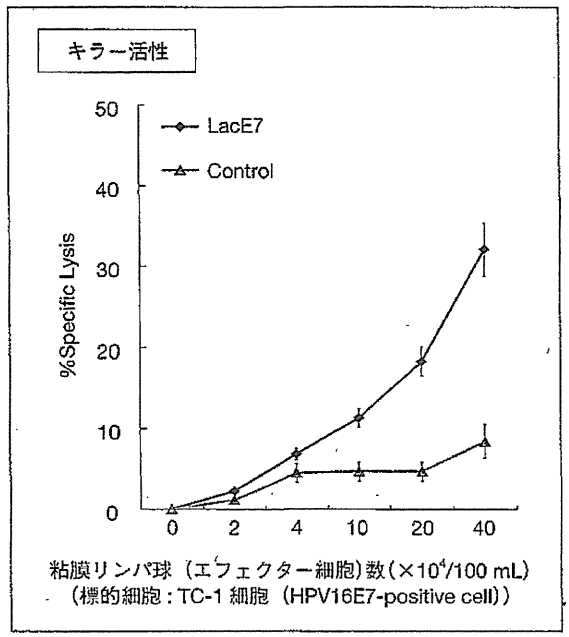

図2　粘膜リンパ球のE7陽性細胞に対するキラー活性
（文献20より引用）

注もしくは筋注による全身性のE7に対する細胞傷害誘導型リンパ球（E7-Th1）を誘導するものであった．いずれの研究でも末梢血中のE7-Th1は良好であった．しかし，子宮頸部局所でのCINに対する治療効果が得られていない．

粘膜免疫を利用した新しいE7標的がんワクチン療法

粘膜内の免疫システムは，全身性免疫とは異なるシステムによって担われている．粘膜免疫システムの要は，粘膜リンパ組織（mucosa-associated lymphoid tissue：MALT）であり，免疫の誘導・メモリーを行っている．しかし，生殖器粘膜にはMALTが存在しない．パイエル板などの腸管粘膜のgut-associated lymphoid tissue GALTが生殖器粘膜のMALTの代わりをしている．筆者らが，子宮頸部の粘膜リンパ球（上皮内リンパ球，intraepithelial lymphocyte：IEL）を解析したところ，子宮頸部リンパ球（CD3-gated）の約25％がインテグリン陽性CCR9陽性（インテグリンとCCR9はGALTにおいてのみ発現するマーカーであり，腸管粘膜由来のリンパ球であることを示す）であり，腸管の粘膜リンパ球が子宮頸部上皮内にホーミングしていることがわかった[19]．

子宮頸部粘膜免疫にとってのメモリー組織であるGALTを直接的に抗原刺激するため，筆者らは経腸管投与によるHPV治療ワクチンを考えた．これまでの海外でのHPV治療ワクチンにはなかった戦略である．そこで注目したのが乳酸菌 *Lactobacillus casei* である．まず乳酸菌食品として広く食経験のある安全性の高い菌種である．もう

一点は，乳酸菌は oral tolerance によって腸管内で免疫寛容を獲得している菌体であり，免疫排除を受けないというメリットがある．筆者らががんワクチンとして用いたのは，*Lactobacillus casei* に HPV16 型 E7（全長）を提示させた HPV16E7 発現乳酸菌である．この E7 発現乳酸菌を加熱処理によって死菌化させたものを製剤化した．

マウスでの経口投与によるに抗 E7 粘膜免疫誘導能では，マウスの腸管粘膜リンパ球に HPV16E7 に特異的な IFNγ 産生細胞数が誘導され，その粘膜リンパ球（インテグリン陽性 T 細胞）には HPV16 陽性上皮細胞に対する細胞傷害（キラー）活性も確認された（**図 2**）．従来の治療的ワクチンで用いられた筋注群・皮下注群と比較すると，粘膜リンパ球における HPV16E7 特異的 IFNγ 産生細胞数は，GLBL101c 経腸管投与群が最も高く，筋注群の 10 倍，皮下注群の 2 倍となった．GLBL101c 経腸管投与群は粘膜免疫誘導に優れていることがわかった[20]．腸管粘膜リンパ球に E7 特異的 IFNγ 産生細胞が誘導されたことから，子宮頸部上皮まで E7 特異的 IFNγ 産生細胞がホーミングすると期待される．そこで，筆者らは，この E7 発現乳酸菌の GMP 製造した経口薬について，子宮頸がん前がん病（CIN3）に対する臨床効果を調べる第Ⅰ/Ⅱa 相臨床試験を実施した．1 g/日内服群では，子宮頸部上皮内リンパ球に HPV16E7 特異的 IFNγ 産生細胞が観察され，かつ CIN2 への退縮が観察され，子宮頸部円錐切除術を回避できている．詳細については，現在行っている最終解析を待って発表する予定である．

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ORIGINAL ARTICLE

Association between carotenoids and outcome of cervical intraepithelial neoplasia: a prospective cohort study

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Abstract
Background It has been suggested that micronutrients such as alpha-tocopherol, retinol, lutein, cryptoxanthin, lycopene, and alpha- and beta-carotene may help in the prevention of cervical cancer. Our aim was to investigate whether serum concentrations and/or dietary intake of micronutrients influence the regression or progression of low-grade cervical abnormalities.
Methods In a prospective cohort study of 391 patients with cervical intraepithelial neoplasia (CIN) grade 1–2 lesions, we measured serum micronutrient concentrations in addition to a self-administered questionnaire about dietary intake. We evaluated the hazard ratio (HR) adjusted for CIN grade, human papillomavirus genotype, total energy intake and smoking status.

The Japan HPV and Cervical Cancer (JHACC) Study Group.

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Results In non-smoking regression subjects, regression was significantly associated with serum levels of zeaxanthin/lutein (HR 1.25, 0.78–2.01, $p = 0.024$). This benefit was abolished in current smokers. Regression was inhibited by high serum levels of alpha-tocopherol in smokers ($p = 0.042$). In progression subjects, a significant protective effect against progression to CIN3 was observed in individuals with a medium level of serum beta-carotene [HR 0.28, 95 % confidence interval (CI) 0.11–0.71, $p = 0.007$), although any protective effect from a higher level of serum beta-carotene was weaker or abolished (HR 0.52, 95 % CI 0.24–1.13, $p = 0.098$). Increasing beta-carotene intake did not show a protective effect (HR 2.30, 95 % CI 0.97–5.42, $p = 0.058$).

Conclusions Measurements of serum levels of carotenoids suggest that regression is modulated by smoking status. Maintaining a medium serum level of beta-carotene has a protective effect for progression; however, carotene intake is not correlated with serum levels of carotenoids.

Keywords Human papillomavirus · Cervical intraepithelial neoplasia · Low-grade squamous intraepithelial lesion · Micronutrients · Carotenoids

Introduction

Persistent infection with human papillomavirus (HPV) may potentially lead to the development of cervical cancer. Most women are exposed to at least one type of genital HPV in their lifetime [1]. HPV infections often cause cervical intraepithelial neoplasia 1 (CIN1) [2]. Only a subset of individuals with CIN1 progress to CIN3 or invasive cervical cancer, suggesting that environmental cofactors are related to cervical carcinogenesis [3–5]. Numerous environmental candidates such as oral contraceptives, parity, smoking status, micronutrient status, nutrient intake, *Chlamydia trachomatis* infection and herpes simplex virus type 2 infection have been investigated as potential cofactors related to progression of CIN.

Much attention has been given to the role of dietary factors and serum micronutrients in the etiology of cervical cancer and CIN. Carotenoids and tocopherols are lipid-soluble micronutrients with potent antioxidant activities and modulatory effects on immunity. Recent publications have reported that the association of carotenoids and tocopherols with reduced risk has not been observed consistently [6–10]; however, these inconsistent results may be due to the study designs. Furthermore, the majority of case–control studies of the associations between micronutrients and outcome of CIN were conducted to assess either dietary intake or circulating micronutrients only [7–9, 11].

Foods are composites of several biologically active dietary components. Micronutrients in foods, as well as other possible anti-carcinogenic compounds such as detoxification enzymes, may have synergistic effects and interact with one another [11–13]. A recent multi-center cohort study reported an association between dietary intake of micronutrients and outcome of CIN. However, this study reported no information about circulating micronutrients [6]. Conversely, some prospective cohort studies reported an association between circulating micronutrient levels and outcome of CIN but no information about dietary intake [14, 15]. Both dietary intake and circulating serum concentrations of micronutrients are important in assessing the role of micronutrients in cervical carcinogenesis. We previously conducted a case–control study including 156 pairs of women with CIN1–3 and matched controls with normal cytology and found an inverse relationship between serum levels of alpha-carotene, lycopene and zeaxanthin/lutein and the risk of CIN development [16]. Because retrospective analysis of previous study findings provides only limited information, we report here the results of a prospective study that was conducted in an attempt to confirm these findings.

Materials and methods

Study design

We used follow-up data from the Japan HPV and Cervical Cancer Study, a prospective non-intervention cohort study conducted to identify determinants of low-grade squamous intraepithelial lesion (LSIL)/CIN regression and progression. Among a total of 570 study subjects with low-grade cervical abnormalities (cytological LSIL and histological CIN1/2) recruited from nine hospitals between 1998 and 2004, 391 women with data concerning serum micronutrients and complete entry questionnaires were enrolled in the present study. Details of the design, methods and primary results have been provided elsewhere [17, 18]. Participants entered the study only after voluntarily giving signed, informed consent. The subjects were routinely followed at 3- to 4-month intervals and received cytology and colposcopy examinations at each visit. To avoid interference of the biopsy procedure on the natural course of the disease, cervical biopsy was performed only when women had HSIL smears and major colposcopic changes that were suggestive of progression to CIN3 or worse. Progression was defined as histological CIN3 lesions or worse, diagnosed on central pathology review. We defined regression as at least two consecutive negative smears and normal colposcopy. Women were regarded as having persistent lesions when they did not have either regression or

progression over the period of follow-up. At enrollment, study subjects were tested for cervical HPV-DNA and circulating serum micronutrients. Information about smoking and dietary intake was obtained from a self-administered questionnaire. Participants were not obliged to answer the questionnaire and their participation was unrelated to their clinical evaluation, treatment or follow-up evaluation. The simplified diet history questionnaire used in the current study had been developed and validated previously [19]. Originally, a prototype diet history questionnaire including 169 traditional Japanese foods and dishes was developed. To alleviate the participants' burden, our simplified diet history questionnaire was developed to employ a stepwise regression method to select from the 169 diet history questionnaire items. This simplified questionnaire was composed of 14 categories: (1) dishes of meat and vegetables; (2) meat (without dishes including vegetables); (3) fish; (4) cereals; (5) eggs and soybean products; (6) vegetables; (7) seaweed; (8) juice; (9) fruits; (10) milk and dairy products; (11) desserts and snacks; (12) pickles; (13) seasoning; and (14) alcoholic beverages. Supplement use was not assessed in this study because of a lack of complete information regarding availability. Because it was impossible to distinguish between intake of alpha- and beta-carotene from the questionnaire, total carotene intake was described. Questions on smoking habits included status (never, former or current smoker) and intensity (number of cigarettes smoked per day).

Circulating micronutrients

Blood was collected in foil-wrapped glass tubes without heparin. Serum was separated by centrifugation at $1{,}000\times g$ for 10 min and stored in the dark at −70 °C prior to sample preparation. Serum levels of retinol, alpha-tocopherol and various carotenoids were determined by a high-pressure liquid chromatography method described previously [21].

Statistical analysis

The association between smoking status and nutrient intake was analyzed by one-way analysis of variance. The association between smoking status and serum micronutrients was analyzed by analysis of covariance. The data were adjusted for age, body mass index (BMI) and alcohol intake frequency. For regression or progression, time to event was measured from the date of the index visit to the date of the visit at which cytological transition to normal or CIN3 was first detected. To estimate the association between the CIN outcomes and circulating serum micronutrients, serum micronutrient tertiles were examined. Hazard ratios (HRs) and 95 % confidence intervals (CIs) for each tertile with reference to the lowest tertile were calculated using a proportional hazard model. For nutrient intake, identical estimation was conducted. The Brinkman Index (BI) was calculated by multiplying the average number of cigarettes smoked per day by the smoking years. We detected HPV-DNA in exfoliated cervical cells by a PCR-based methodology described previously [20]. HPV DNA was amplified by PCR using consensus-primers (L1C1/L1C2 + L1C2M) for the HPV L1 region. HPV genotypes were identified by a restriction fragment-length polymorphism (RFLP) PCR method that has been shown to identify at least 26 genotypes of genital HPV [18]. HRs were adjusted for potential confounders, including CIN grade, HPV genotype, age, total energy intake and smoking status. Statistical analyses were performed using Stata statistical software, release 11.1 (Stata Corporation; College Station, TX, USA).

Results

Of the 570 women enrolled in the parent study, 391 met the eligibility requirements of the current study for tests of serum micronutrients and completion of entry questionnaires. Of these, 329 and 62 women were diagnosed as CIN1 and CIN2, respectively. The mean age of the women was 36.3 years (median 36.0, range 19–54). Of the 391 women, regression, persistence and progression occurred in 218, 135 and 38, respectively.

Influence of smoking status on circulating levels and intake of micronutrients

At enrollment, 190 women had never smoked, while 142 women were current smokers (BI >100). Data from three women were lost and the remaining 56 women were past smokers. We found a 22 and 10 % decrease in carotene and vitamin E intake in current smokers compared with non-smokers, respectively (Table 1). Among the three groups, there was a significant difference in the intake of fiber, calcium, carotenes, vitamin A, vitamin C and vitamin E. As shown in Table 2, current smokers had significantly lower serum levels of alpha-carotene, beta-carotene and cryptoxanthin compared with non-smokers. Smokers had marginally lower levels of lycopene. Retinol, zeaxanthin/lutein and alpha-tocopherol were not related to smoking status.

The effects of serum micronutrients and nutrient intake in regression subjects

Significantly more inhibition of regression was observed in women in the middle tertiles of serum alpha-tocopherol

Table 1 Relationship between estimated daily nutrient intake and tobacco smoking status

Nutrient intake per day	Non smokers ($N = 190$)		Past smokers ($N = 56$)		Current smokers ($N = 142$)		p value
	Mean	SD	Mean	SD	Mean	SD	
Total energy intake (kcal)	2,220.1	576.1	2,221.6	679.7	2,149.1	574.9	0.520
Protein intake (g)	85.2	26.2	85.2	31.0	79.4	27.3	0.127
Fat intake (g)	60.2	21.9	62.9	27.2	59.0	22.6	0.566
Carbohydrate intake (g)	329.5	78.3	325.2	85.6	315.2	74.6	0.255
Fiber intake (g)	5.3	1.9	5.2	2.0	4.6	1.8	0.004
Calcium intake (mg)	740.8	292.2	738.3	337.6	620.9	274.2	0.001
Retinol intake (μg)	284.6	219.1	302.4	176.9	331.2	624.7	0.597
Carotene intake (μg)	4,943.5	2,439.7	4,856.3	2,532.1	3,866.8	2,083.5	0.000
Vitamin A intake (IU)	3,430.6	1,587.5	3,424.3	1,546.9	2,954.2	2,197.4	0.049
Vitamin C intake (mg)	134.0	65.6	133.3	65.9	113.4	56.4	0.008
Vitamin D intake (IU)	76.4	48.8	69.3	40.6	66.9	53.7	0.213
Vitamin E intake (mg)	8.4	2.8	8.3	3.2	7.5	2.7	0.021
Salt intake (g)	13.5	4.1	13.7	4.8	12.8	4.5	0.291
Cholesterol intake (mg)	323.7	122.6	322.9	160.2	304.7	137.5	0.412

Analysis of variance was used to examine the differences in the mean values of factors among groups

SD standard deviation

Table 2 Relationship between serum micronutrients and tobacco smoking status

	Non-smoker ($N = 190$)		Past smoker ($N = 56$)		Current smoker ($N = 142$)		P value
	Adjusted mean	95 % CI	Adjusted mean	95 % CI	Adjusted mean	95 % CI	
Serum retinol (μg/dL)	59.23	56.42–62.04	59.70	54.59–64.81	60.88	57.24–64.51	0.695
Serum α-carotene (μg/dL)	9.70	8.58–10.82	7.47	5.43–9.51	7.23	5.78–8.68	0.003
Serum β-carotene (μg/dL)	58.05	50.77–65.33	46.61	33.36–59.85	41.02	31.60–50.44	0.003
Serum zeaxanthin/lutein (μg/dL)	54.93	50.77–59.09	54.06	46.50–61.62	49.88	44.50–55.26	0.205
Serum cryptoxanthin (μg/dL)	31.19	25.61–36.76	23.61	13.46–33.76	21.27	14.05–28.49	0.03
Serum lycopene (μg/dL)	30.00	26.76–33.22	34.68	28.80–40.55	27.23	23.04–31.41	0.06
Serum α-tocopherol (μg/dL)	881.68	817.51–945.84	953.15	836.40–1,069.91	873.56	790.50–956.63	0.414

Analysis of covariance was used to examine the differences in the mean concentrations of the serum levels of micronutrients that are related to the effect of the smoking status. The data were adjusted for age (20–29, 30–39, or 40–54 years), BMI and alcohol intake frequency (0, 1–6, 7/week)

(HR 0.68, 95 % CI 0.49–0.95) as compared with women in the lower tertiles, but the linear trend was not statistically significant ($p = 0.882$). From the questionnaire, high-load intake of retinol significantly inhibited the regression (adjusted model: HR 0.59, 95 % CI 0.40–0.89) but the linear trend was not significant (Table 3).

Because serum levels of most carotenoids were low and carotene intake was small in smokers, the regression group was sub-analyzed stratifying by smoking status (never or current smokers) as shown in Tables 4 and 5. In non-smokers (Table 4), regression was observed in women in the upper tertiles of serum zeaxanthin/lutein (HR 1.25, 95 % CI 0.78–2.01) as compared with women in the lower and middle tertiles, and the linear trend was statistically significant ($p = 0.024$). In current smokers, this was statistically abolished as shown in Table 5. In current smokers, a significant inhibition of regression was observed in women in the middle tertiles for serum alpha-tocopherol (HR 0.53, 95 % CI 0.27–0.94) as compared with women in the lower tertiles, and the linear trend was significant ($p = 0.042$) in the adjusted model (Table 5).

Effect of serum micronutrients and nutrient intake in progression subjects

In Table 6, a significant inverse relationship was observed in subjects with a medium level of serum beta-carotene (HR 0.28, 95 % CI 0.11–0.71, $p = 0.007$), although these

Table 3 HR of regression from entire 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	0.812
Low (<55.2)	128	1,715.6	74	62.5 (53.6–71.4)	1		1	
Medium (55.2–67.9)	132	1,689.8	77	63.2 (54.4–72.0)	1.06 (0.77–1.46)	0.709	1.19 (0.86–1.65)	0.301
High (>67.9)	131	1,763.5	67	57.8 (48.6–67.4)	0.87 (0.62–1.21)	0.399	0.87 (0.62–1.22)	0.423
Serum α-carotene							*p* for trend	0.472
Low (<5.1)	127	1,654.9	71	60.9 (51.9–70.0)	1.00		1.00	
Medium (5.1–9.7)	133	1,750.0	68	57.3 (48.2–66.8)	0.91 (0.65–1.27)	0.574	1.00 (0.71–1.41)	0.984
High (>9.7)	131	1,764.0	79	65.2 (56.5–73.9)	1.04 (0.75–1.43)	0.828	1.26 (0.89–1.80)	0.19
Serum β-carotene							*p* for trend	0.095
Low (<28.3)	129	1,679.7	66	56.7 (47.7–66.2)	1.00		1.00	
Medium (28.3–57.6)	131	1,755.9	75	62.7 (53.8–71.6)	1.10 (0.79–1.53)	0.581	1.17 (0.83–1.66)	0.364
High (>57.6)	131	1,733.3	77	64.0 (55.2–72.9)	1.12 (0.80–1.56)	0.511	1.34 (0.93–1.93)	0.115
Serum zeaxanthin/lutein							*p* for trend	0.235
Low (<42.9)	130	1,645.9	76	62.7 (53.8–71.6)	1.00		1.00	
Medium (42.9–57.3)	130	1,803.1	70	58.1 (49.2–67.2)	0.85 (0.62–1.18)	0.341	0.97 (0.69–1.36)	0.868
High (>57.3)	131	1,719.9	72	63.5 (54.2–72.7)	0.89 (0.65–1.23)	0.488	1.05 (0.75–1.48)	0.768
Serum cryptoxanthin							*p* for trend	0.215
Low (<11.2)	129	1,659.5	74	63.9 (54.8–73.0)	1.00		1.00	
Medium (11.2–22.1)	130	1,754.7	67	56.8 (47.8–66.2)	0.87 (0.62–1.21)	0.406	0.91 (0.65–1.28)	0.592
High (>22.1)	132	1,754.7	77	63.1 (54.3–71.9)	0.99 (0.72–1.37)	0.974	1.07 (0.76–1.51)	0.694
Serum lycopene							*p* for trend	0.638
Low (<19.8)	129	1,713.7	69	58.6 (49.7–67.9)	1.00		1.00	
Medium (19.8–35.8)	131	1,780.3	79	66.3 (57.4–75.0)	1.07 (0.78–1.48)	0.67	1.07 (0.76–1.49)	0.705
High (>35.8)	131	1,674.9	70	58.5 (49.4–67.8)	1.02 (0.73–1.42)	0.914	1.08 (0.77–1.52)	0.662
Serum α-tocopherol							*p* for trend	0.882
Low (<753.0)	128	1,535.8	82	67.3 (58.7–75.6)	1.00		1.00	
Medium (753.0–983.9)	132	1,896.8	66	54.7 (45.9–64.0)	0.66 (0.48–0.91)	0.011	0.68 (0.49–0.95)	0.025
High (>983.9)	131	1,736.3	70	62.8 (53.2–72.3)	0.74 (0.54–1.01)	0.062	0.78 (0.56–1.09)	0.142
Retinol intake							*p* for trend	0.322
Low (<190.2)	130	1,555.8	74	62.8 (53.6–72.0)	1.00		1.00	
Medium (190.2–313.1)	130	1,755.6	74	63.3 (54.0–72.0)	0.89 (0.65–1.23)	0.484	0.76 (0.54–1.07)	0.12
High (>313.1)	131	1,857.5	70	57.8 (49.0–66.9)	0.80 (0.57–1.10)	0.172	0.59 (0.40–0.89)	0.011
Carotene intake							*p* for trend	0.325
Low (<3,281.4)	130	1,639.3	70	59.8 (50.6–69.1)	1.00		1.00	
Medium (3,281.4–5,042.8)	131	1,812.8	72	61.6 (52.5–64.7)	0.92 (0.66–1.28)	0.637	0.90 (0.63–1.28)	0.557
High (>5,042.8)	130	1,716.8	76	62.2 (53.5–71.0)	1.03 (0.74–1.42)	0.869	0.97 (0.65–1.46)	0.89
Vitamin A intake							*p* for trend	0.546
Low (<2,398.8)	130	1,601.8	70	61.5 (62.5–74.6)	1.00		1.00	
Medium (2,398.8–3,466.7)	131	1,834.7	72	59.7 (51.7–64.7)	0.90 (0.65–1.25)	0.541	0.91 (0.64–1.29)	0.599
High (>3,466.7)	130	1,732.4	76	62.6 (53.9–71.4)	1.01 (0.73–1.40)	0.948	0.93 (0.61–1.42)	0.727
Vitamin E intake							*p* for trend	0.147
Low (<6.7)	130	1,610.2	68	57.4 (48.3–66.7)	1.00		1.00	
Medium (6.7–8.7)	130	1,897.1	71	59.4 (50.5–68.5)	0.90 (0.64–1.25)	0.521	0.95 (0.66–1.39)	0.807
High (>8.7)	131	1,661.6	79	65.9 (57.1–74.6)	1.11 (0.80–1.54)	0.519	0.88 (0.54–1.43)	0.601

Cox's proportional hazard model showing the hazard ratio for regression in a cumulative 24-month period. The adjusted model was calculated by CIN grade (initial biopsy results; CIN1 or CIN2), HPV genotypes (HPV16/18/31/33/35/42/52/59, other high-risk types, low-risk types, or HPV negative) [17, 18], age, total calorie intake and smoking status (Brinkman index >100). The units of micronutrients are expressed as μg/dL

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

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

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

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.

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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Sequential effects of the proteasome inhibitor bortezomib and chemotherapeutic agents in uterine cervical cancer cell lines

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Abstract. Although the prognosis of uterine cervical cancer has improved due to the advances of treatment modalities, survival of recurrent or metastatic cervical cancer remains poor. Cisplatin is an effective radiosensitizer, but its single agent activity in recurrent cervical cancer is disappointing. Inactivation of tumor suppressors through ubiquitin-mediated degradation by human papillomavirus is known to be a critical step in the carcinogenesis of uterine cervix. Bortezomib, a selective inhibitor of the proteasome, has been shown to inhibit the growth of several solid tumors. To determine the role of bortezomib in cervical cancer as a chemotherapeutic agent, we studied its biological properties. Bortezomib efficiently inhibited the proteasomal activities in cervical cancer cells, and an increased expression of tumor suppressors such as p53, hDlg and hScrib became evident. In addition, sequential or concomitant treatment of bortezomib and cisplatin stimulated the expression of p53, hScrib and p21 and the stimulation was markedly influenced by the order of drugs in HeLa cells. We further confirmed that the concomitant use of bortezomib and cisplatin has synergistic inhibitory effects on the growth of xenograft tumors derived from HeLa cells. Our data establish the possibility that the concomitant use of bortezomib and cisplatin could be an alternative choice in cases resistant to conventional chemotherapy, and sequential effects must be considered for advanced and therapy-resistant cervical cancer patients.

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Introduction

Cancer of the uterine cervix is the second most common cause of gynecologic cancer mortality worldwide, and it is reported that cervical cancer affected 493,243 women worldwide in 2002 (1). It remains a health threat with estimated incidence and mortality rates of 12,710 and 4,290 in 2011, respectively, in the United States (2). Cervical cancer is now considered a preventable disease (3), but it is important to note that cervical cancer affects young women at a higher incidence. Treatment paradigms in the primary management of cervical cancer are well established. Early stage patients are treated surgically and women with locally advanced disease are managed with concomitant cisplatin chemoradiotherapy. However, the prognosis of patients with metastatic, recurrent, or persistent cervical cancer remains poor with a 1-year survival rate between 15-20% (4). In addition, chemotherapy has not led to major improvements in clinical outcome and is associated with high rates of severe toxicities. Advanced cervical cancer is associated with significant morbidities such as renal failure, complex fistulas and painful bone metastases. Therefore, improvement of systemic chemotherapy is crucial and new regimens should be further developed.

Persistent infection with an oncogenic-type human papillomavirus (HPV) is thought to be a prerequisite for the development of cervical cancer. The two HPV oncogenes, E6 and E7, are required for efficient immortalization of primary epithelial keratinocytes. The E6 proteins form a complex with p53 (5,6), and subsequent disruption of multiple functions of p53 is an important step in cervical carcinogenesis (7). E6 also affects the function of tumor suppressors involved in apoptosis, cell cycle regulation, and tissue polarity, including two human homologues of *Drosophila* neoplastic tumor suppressors, hDlg and hScrib (8,9).

Recent evidence indicates that rapidly proliferating cells, particularly cancer cells, have a greater requirement for proteasomal activity and a greater sensitivity to the proteasome inhibitor compared to normal cells (10). Bortezomib, a proteasome inhibitor, has been approved by the United

States Food and Drug Administration for the treatment of multiple myeloma, and bortezomib has shown *in vitro* and *in vivo* activity against solid tumors, including prostate, pancreatic and colon cancer (11). In gynecologic cancers, several investigators have reported implicative roles of bortezomib for the treatment of human cervical (12-16) and ovarian cancer (17).

In this study, we report that sequential or concomitant use of bortezomib with cisplatin markedly induces apoptosis and inhibition of growth in cultured cervical cancer cells and xenograft. Bortezomib may have pre-clinical activity in cisplatin-resistant tumors and may have synergic activity when combined with cisplatin in HeLa cells. These effects may have an important clinical implication to maximize the stabilization of tumor suppressors (18).

Materials and methods

Chemicals and antibodies. Bortezomib (VELCADE, formerly known as PS-341) was kindly provided by Millennium Pharmaceuticals (Cambridge, MA, USA). Cisplatin, carboplatin and paclitaxel were from Bristol-Myers Squibb (Princeton, NJ, USA). Bortezomib, cisplatin, carboplatin and paclitaxel were dissolved in dimethyl sulfoxide and the final concentration of dimethyl sulfoxide never exceeded 0.05%.

Anti-hScrib, anti-pRb (Ser 795), anti-p53 and anti-p21 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rabbit polyclonal antibody was anti-Noxa (AnaSpec, Inc., San Jose, CA, USA). Mouse monoclonal antibody was anti-α-Tubulin (Calbiochem, EMD Biosciences, Inc., La Jolla, CA, USA). Alexa Fluor 488-conjugated donkey anti-mouse IgG (A-21202) and Alexa Fluor 555-conjugated goat anti-rabbit IgG (A-21428) were purchased from Invitrogen (Carlsbad, CA, USA).

Cell culture. HeLa (CCL-2) and CaSki (HB-8307) uterine cervical cancer cell lines were purchased from the American Type Culture Collection (Manassas, VA, USA) and grown in DMEM supplemented with 10% fetal bovine serum.

Sequential and simultaneous treatment regimens. To determine the effect of sequence difference on cellular response, cells were treated with bortezomib (100 nM), carboplatin (250 μM), paclitaxel (10 μM) and cisplatin (500 μM). HeLa and CaSki cells were seeded and allowed to adhere for 24 h. For the sequential treatment, after 12 h of initial treatment, the medium was changed to fresh medium containing the other treatment. For the simultaneous treatment, after an initial 12 h in medium, cells were treated with fresh medium containing the same drugs. The control cell medium was changed at similar time points. After the second 12-h treatment, the cells were harvested for flow cytometric analysis or western blotting. Therefore, all groups received the same duration of exposure to each agent and the assays were performed at the same point following the final treatment.

Cell viability test. Viability of HeLa and CaSki cells was examined using the CellTiter 96 Aqueous One Solution Cell Proliferation Assay kit (Promega Corp., Madison, WI, USA), as previously described (19).

Pulse chase analysis of p53 and hScrib. The culture medium of HeLa cells was replaced with Met/Cys-free DMEM for 2 h and pulsed with 20 μCi/ml of EasyTag™ EXPRESS^{35}S (Perkin-Elmer Life Sciences, Boston, MA, USA) for 1.5 h. The ^{35}S-labeled protein was chased with or without bortezomib (100 nM). Cells were harvested at the indicated time point, lysed and electrophoresed.

Western blotting. Cultured cells and mouse xenograft tissues were harvested and soluble protein was extracted. The procedure of western blotting and subsequent immunoblot was performed as previously described (20).

Flow cytometric analysis. To determine the apoptosis rate, cells were grown in DMEM and treated with chemotherapeutic drugs. Bortezomib, cisplatin, carboplatin and paclitaxel treatments in 12-h increments over a 24-h period were as described. Following treatment, cells were harvested and stained with Annexin V-FITC and propidium iodide (PI) according to the manufacturer's protocol (BD Biosciences, Bedford, MA, USA). The percentage of specific apoptosis was analyzed on FACSCalibur and calculated by CELLQuest Pro software (BD Biosciences).

Quantification of the synergism of bortezomib with cisplatin in the manner of sequential or simultaneous administration (Chou-Talalay assay). For the Chou-Talalay assay, experiments were carried out as previously described (21). Dose-response curves and 50% effective dose values (ED_{50}) were obtained, and fixed ratios of drugs and mutually exclusive equations used to determine combination indices (CI) (22). The potency of the combination was calculated with the CalcuSyn software (Biosoft, Ferguson, MO, USA). CI<1, CI=1, and CI>1 indicate synergistic, additive and antagonistic interactions, respectively.

Tumor growth suppression in vivo. Athymic C.B-17/Icr-scid Jcl mice 5-7 weeks of age (CLEA Japan, Inc., Tokyo, Japan) were maintained in an SPF facility according to the institutional guidelines, and experiments were conducted under an approved animal protocol of The University of Tokyo. Subcutaneous xenograft tumors were established by the injection of cell suspension of $1x10^7$ HeLa and CaSki cells. After the appropriate tumors were formed, the mice were sacrificed. The tumors were removed, cut into 3-mm sections and transplanted subcutaneously into other mice. Mice were randomly assigned to one of the four treatment regimens: saline (control), cisplatin [intraperitoneal (i.p.) injection of cisplatin at a dose of 6 mg/kg in a volume of 0.5 ml (23)], bortezomib [i.p. injection of bortezomib at a dose of 1 mg/kg in a volume of 0.5 ml (24)], and bortezomib followed by cisplatin 8 h later in a combined volume of 0.5 ml. Each treatment group consisted of 6 mice. Tumors were measured and the volume of these tumors was calculated using the formula; Volume (mm^3) = [(major axis) x (minor axis)^2]/2. After 4 weeks of treatment, the mice were sacrificed and subjected to the analysis.

Immunofluorescence. The mouse xenograft tumors were frozen in OCT compounds (Sakura Finetek Japan Co., Ltd., Tokyo, Japan). The embedded tissues were cut (6 μm) and cryostat sections were recovered and fixed with PBS containing

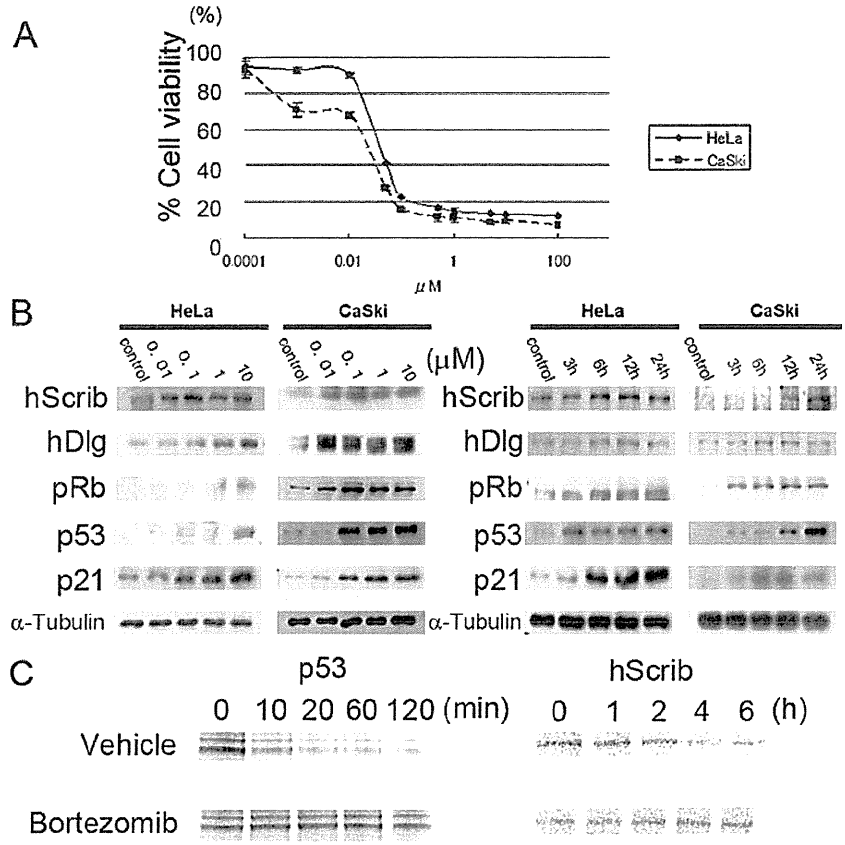

Figure 1. Biological effects of bortezomib on human cervical cancer cell lines and stabilization of tumor suppressors. (A) HeLa and CaSki cells were exposed to bortezomib for 48 h and cell viability was determined by MTS assay. Each point represents the means ± SD of 3 independent experiments. (B) The effect of bortezomib on the expression of tumor suppressor proteins. (1) HeLa and CaSki cells were exposed to various concentrations of bortezomib for 12 h (left panel). (2) Cells treated by 100 nM of bortezomib were harvested at the indicated time points (right panel). The expression levels of hScrib, hDlg, pRb, p53 and p21 were analyzed by western blotting. (C) Pulse chase analysis of p53 and hScrib. p53 and hScrib proteins were ^{35}S-labeled in HeLa cells and the amount of labeled proteins were chased in the presence and absence of bortezomib. In the presence of bortezomib, the expression of p53 and hScrib remained unaffected during the observed time.

4% paraformaldehyde. After blocking, the cells were sequentially incubated with anti-p53 or anti-hScrib antibodies and appropriate secondary antibodies. The slides were briefly counterstained and analyzed under the fluorescence microscope (Olympus BX50; Olympus, Tokyo, Japan). Apoptotic cells were detected by DeadEnd™ Fluorometric TUNEL System (Promega Corp.) in the mouse xenograft tumors.

Statistical analysis. Data represent the means ± SD or SEM from at least 3 independent experiments. Statistical analyses were performed by one-way ANOVA with post-hoc test for multiple comparisons by using StatView software (SAS Institute Inc., Cary, NC, USA). A P-value <0.05 was considered to indicate statistically significant differences.

Results

Effect of bortezomib on cellular viability and stabilization of tumor suppressor proteins targeted for degradation by HPV E6 and E7 in human cervical cancer cell lines. We used MTS assay to determine the effect of bortezomib on cell viability in HeLa and CaSki cells (Fig. 1A). The approximately estimated IC_{50} of bortezomib in HeLa and CaSki cells was 100 nM. The expression of p53 increased by the exposure to bortezomib in a dose-dependent manner (Fig. 1B left panel) and in a time-dependent manner (Fig. 1B right panel). As a result, the expression of p21 also increased. Elevated expression of PDZ domain-containing scaffolding proteins (hScrib and hDlg) was shown by the exposure to bortezomib (Fig. 1B). The expression of pRb decreases by the proteasome system during cervical carcinogenesis (25), and the expression of pRb was remarkably stimulated by the addition of bortezomib, particularly in CaSki cells (Fig. 1B). These data were translated as the antitumorigenic properties of bortezomib and we further confirmed whether p53 and hScrib are stabilized in the presence of bortemozib by the pulse-chase analysis. As expected, p53 and hScrib expression remained unaffected in the presence of bortezomib in HeLa cells (Fig. 1C).

Effect of bortezomib and chemotherapeutic agents on cervical cancer cells. The effect of bortezomib and chemotherapeutic drugs on the induction of apoptosis was analyzed by the flow cytometric analysis (Fig. 2A). As a single agent, bortezomib (lane 2) possessed a significant ability to induce apoptosis compared to cisplatin, carboplatin, and paclitaxel (lanes 3, 4 and 5, respectively), and the rate of apoptosis was superior in CaSki cells. The sequential and simultaneous combination of bortezomib with cisplatin was identified to be the most