Journal of Pathology
J Pathol 2003; **201**: 487–495.
Published online 25 September 2003 in Wiley InterScience (www.interscience.wiley.com). **DOI:** 10.1002/path.1419

Original Paper

Mechanisms of inactivation of the *p16*^{INK4a} gene in leiomyosarcoma of soft tissue: decreased p16 expression correlates with promoter methylation and poor prognosis

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Received: 27 November 2002
Revised: 12 February 2003
Accepted: 26 March 2003

Abstract

The *p16*^{INK4a} tumour suppressor gene, encoding p16 protein, plays a crucial role in regulation of the G1 cell-cycle phase. To investigate the potential role of p16 in soft tissue leiomyosarcoma (LMS), an immunohistochemical analysis was performed of 77 LMSs for p16 expression. Decreased expression of the p16 protein was identified in 25 of 77 LMSs (32%). Decreased expression of p16 correlated significantly with large tumour size ($p = 0.0038$). In a univariate analysis, large tumour size and decreased expression of p16 were statistically significant adverse prognostic factors ($p = 0.025$ and $p = 0.0021$, respectively). In a multivariate analysis including conventional clinicopathological parameters, decreased expression of p16 protein was revealed as the only independent unfavourable prognostic factor ($p = 0.012$). To elucidate the mechanisms of inactivation of the *p16*^{INK4a} gene, 49 LMSs for which genomic DNA was available were examined; analysis for homozygous deletion, mutation, and promoter hypermethylation was conducted using differential PCR, PCR–SSCP, and methylation-specific PCR, respectively. Promoter hypermethylation was detected in 11 of 49 LMS cases (22%); homozygous deletion was detected in 3 of 49 cases (6%); and mutation was not recognized in any of the cases studied. Eight of 15 cases (53%) with decreased expression of p16 protein revealed methylation of the *p16*^{INK4a} gene promoter. Promoter hypermethylation correlated closely with decreased expression and poor prognosis ($p = 0.0014$ and $p = 0.0088$, respectively). These results suggest that decreased expression of p16 protein can be considered as an independent reliable prognostic parameter in patients with soft tissue LMS. Furthermore, promoter methylation was more frequent than either homozygous deletion or mutation in this tumour, and promoter methylation was also shown to have a strong association with inactivation of the *p16*^{INK4a} gene. **Copyright © 2003 John Wiley & Sons, Ltd.**

Keywords: **leiomyosarcoma; *p16*^{INK4a}; promoter hypermethylation; homozygous deletion; immunohistochemistry**

Introduction

The *p16*^{INK4} gene is located on the short arm of chromosome 9, region 9p21, and encodes for a nuclear protein that can block cell-cycle progression by effectively inhibiting the kinase activity of CDK4/6, exerting negative control on cell proliferation [1–3]. In the absence of a functional p16 protein, CDK4 binds to cyclin D and phosphorylates pRb, releasing E2F and stimulating cell-cycle progression. Such activity has been reported in many types of malignancy. It has been suggested that decreased p16 expression correlates with tumour progression and with decreased survival among patients with various types of carcinoma [4–7]. Similarly, decreased p16 expression has been associated with decreased survival in patients with paediatric osteosarcoma [8]. Loss of functional *p16*^{INK4a} genes is frequent in many types of carcinoma and in other malignancies [9,10], and this loss may be due to homozygous deletion [11–14], point mutation [15], or methylation of the promoter region [16–18]. Hypermethylation of the *p16*^{INK4a} gene promoter region, especially when involving CpG-rich areas known as CpG islands, is associated with loss of transcription [19], and has also been demonstrated in some sarcomas [20–26]. The *p16*^{INK4a} gene has been reported to be altered in some sarcomas; however, the frequency and the mechanism for inactivation of the *p16*^{INK4a} gene are not well understood in mesenchymal neoplasms, including leiomyosarcoma (LMS). Furthermore, the association between abnormalities of *p16*^{INK4a} and prognosis in LMS patients remains controversial [26–28].

In the present study, we first examined the immunohistochemical expression of p16 in a large series of

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soft tissue LMSs. We next sought to determine the mechanisms for inactivation of the $p16^{INK4a}$ gene in LMS of soft tissue, because we found that decreased expression of p16 protein was the only independent prognostic factor, as determined by a multivariate survival analysis that included conventional clinicopathological parameters. We found that homozygous deletion and mutation of the $p16^{INK4a}$ gene were rare events. However, hypermethylation of the $p16^{INK4a}$ gene promoter was a frequent event that could account for inactivation of the $p16^{INK4a}$ gene in approximately half of the cases.

Materials and methods

Tumour samples

Seventy-seven formalin-fixed, paraffin wax-embedded tissue samples from 77 patients with leiomyosarcoma (LMS) of soft tissue were collected from the soft tissue tumour files of the Department of Anatomic Pathology, Graduate School of Medical Sciences, Kyushu University, Japan, and were used for the immunohistochemical analysis of p16. The paraffin wax-embedded tissues had been fixed in 10% formaldehyde. The diagnoses of all cases included in this study were based on histological examination with haematoxylin and eosin (H&E) staining, and smooth muscle differentiation was confirmed by immunohistochemical positivity for desmin, muscle actin, and alpha-smooth muscle actin. In addition, the expression of c-kit, a gastrointestinal stromal tumour marker, was not identified in any of the 77 cases. Of these 77, 49 (paraffin-embedded tissues, 33 cases; frozen materials, 16 cases) in which the DNA was of good quality were used for further molecular analyses.

Clinicopathological analysis

The clinicopathological parameters investigated in this study were classified as follows: age ($\geq$60 years versus <60 years); location (abdominal versus extra-abdominal); size ($\geq$5 cm versus <5 cm); mitotic rate ($\geq$20 versus <20); AJCC stage (stages I and II versus stages III and IV); FNCLCC grade (grade 1 versus grades 2 and 3); and histological subtype (pleomorphic versus other subtypes). With regard to the location of the tumours, samples were divided into two groups according to previously described criteria, ie cases in which the tumours were located at deep sites, such as the retroperitoneum or abdominal cavity (abdominal type), and cases in which the tumours arose at superficial sites, such as the skin, subcutis, and skeletal muscles (extra-abdominal type) [29]. The mitotic rate was evaluated by counting the number of mitotic figures in ten high-power fields (HPFs). The histological tumour grade was evaluated according to the grading system of the French Federation of Cancer Centers (FNCLCC) [30]. Whenever possible, cases were also evaluated according to the new American Joint Committee on Cancer (AJCC) staging system [31]. Survival data were available for 60 of the 77 cases, with a follow-up period ranging from 14 to 258 (median 58.4) months.

Immunohistochemistry

Immunohistochemical analyses were performed using a mouse IgG monoclonal antibody against p16 (1 : 50; Santa Cruz Biotechnology, USA). Four-micrometre-thick histological sections were cut, mounted on glass slides coated with 3-aminopropyltriethoxysilane, and air-dried overnight at room temperature. The sections were deparaffinized in xylene and dehydrated in ethanol. After dehydration, the endogenous peroxidase was blocked with methanol containing 3% H_2O_2 for 30 min. For staining with the above antibody, specimens were pretreated with citrate buffer (0.01 mol/l citric acid, pH 6.0) four times, each for 5 min at 100 °C in a microwave oven. Sections were incubated with the primary antibody at 4 °C overnight, followed by staining with a streptavidin–biotin–peroxidase kit (Nichirei, Tokyo, Japan). The sections were then finally reacted in a 3,3′-diaminobenzidine, peroxytrichloride substrate solution; counterstained with haematoxylin; and then mounted. Each case was scored for p16 immunoreactivity using previously published criteria [4,9,32]. For example, if the neoplastic nuclei were stained throughout the tumour, the expression of p16 was considered to be normal. However, if the neoplastic nuclei failed to stain in all areas, although admixed non-neoplastic cells were stained, or if there was an absence of stained neoplastic nuclei in certain areas, with positive staining of the admixed non-neoplastic nuclei, the expression of p16 in these neoplasms was judged to be decreased. Tonsil tissue was used as an external positive control. As a negative control, the primary antibody was omitted.

DNA extraction from tumour tissue

Genomic DNA was extracted using standard proteinase K digestion and phenol/chloroform extraction methods. To avoid contamination of the DNA with normal tissue, manual microdissection was performed to extract tumour DNA from formalin-fixed, paraffin wax-embedded tissues. When using frozen material, we confirmed that there was no contamination by normal tissue in each sample by H&E staining. In each sample, we selected a lesion with high cellularity for the PCR assay.

Differential PCR assay for p16

The differential PCR method for detecting homozygous deletions of *p16* was based on a modification of a reported method using the *β-actin* gene as an internal control [33]. The sequences of the primers are summarized in Table 1; the PCR conditions were the same as those previously described

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Table 1. Primers used for the analysis of homozygous deletion, mutation, and methylation of the *p16*^{INK4} gene

Determination	Primer sequence (5′ to 3′) Sense	Antisense	Product size (bp)	Annealing temperature (°C)
Differential PCR				
p16 exon 1	GAGCAGCATGGAGCCTTC	AATTCCCCTGCAAACTTCCGT	204	60
Beta-actin	CTGTGGCATCCACGAAACTA	AGGAAAGACACCCACCTTGA	187	60
PCR–SSCP				
p16 exon 1	GAGCAGCATGGAGCCTTC	AATTCCCCTGCAAACTTCCGT	204	60
p16 exon 2A	CTGGCTCTGACCATTCTGT	AGCACCACCAGCGTGTCC	171	58
p16 exon 2B	GACCCCGCCACTCTCACC	AGGTACCGTGCGACATCGC	170	58
p16 exon 2C	GATGCCTGGGGCCGTCT	CAGGGTACAAATTCTCAGAT	169	55
p16 exon 3	GTAGGGACGGCAAGAGA	ACCTTCGGTGACTGATG	159	55
p16 MP1	GAGCAGCATGGAGCCTTCGGCTGACCGG		204	60
p16 MP2	GAGCAGCATGGAGCCTTCGGCTGACTGAC		204	60
MSP				
p16M	TTATTAGAGGGTGGGGCGGATCGC	GACCCCGAACCGCGACCGTAA	150	65
p16U	TTATTAGAGGGTGGGGTGGATTGT	CAACCCCAAACCACAACCATAA	151	60

M = methylated; U = unmethylated; MP1 = mutated forward primer 1; MP2 = mutated forward primer 2.

[34]. Human genomic DNA (Clontech), which was confirmed to contain the same base sequences as those of Genebank (Accession No AF000730-734), was used as a normal control for each PCR and for the subsequent reactions. We also confirmed that no contamination had occurred in any of the PCR analyses, or in any of the subsequent reactions by using distilled water. After amplification, 10 µl of the PCR products were electrophoresed through an 8% polyacrylamide gel and the intensities of the DNA products were quantified by National Institutes of Health (NIH) Imaging software Ver 1.56. Values less than 25% for the target gene/internal control ratio were considered to represent a homozygous deletion [33,34].

Mutation analysis of the *p16*^{INK4A} gene by polymerase chain reaction–single-strand conformation polymorphism (PCR–SSCP)

Mutation analysis of the *p16*^{INK4A} gene was performed from exon 1 to exon 3. The sequences of the primers are summarized in Table 1. PCR was carried out for 35 cycles after the first denaturation at 94 °C for 1 min (94 °C for 1 min, the annealing temperature listed in Table 1 for 30 s, and 72 °C for 1 min). PCR products were electrophoresed through a 2.0% agarose gel with ethidium bromide. To validate the sensitivity of the SSCP conditions used in this study, we made two artificially mutant PCR products, as mutations of the *p16*^{INK4A} gene have been reported to be rare and an appropriate positive control was therefore not available. In brief, we amplified human genomic DNA by using two different kinds of point-mutated forward primers instead of primers of normal sequence. The sequences for the point-mutated forward primers are shown in Table 1. The PCR products were electrophoresed on a 2% agarose gel and then cut from the gel. The products obtained were re-amplified by using primers of normal sequence and then the amplified products were used for SSCP analysis. As a result, we successfully identified the aberrantly migrating bands for both of the point-mutated forward primers, as shown in Figure 6. We therefore considered the conditions used for the SSCP to be appropriate. DNA bands were cut from the gels and purified using a SUPREC tube (TAKARA Biomedicals, Japan); the products were re-amplified for 15 cycles. SSCP was performed as previously described [35]. To increase the quantity of DNA prior to sequencing, extra bands, which appeared to be aberrantly migrating, were excised from the SSCP gels and re-amplified for 25 cycles under the same conditions. The samples were analysed for sequencing after the subsequent reaction.

Bisulphite modification and methylation-specific PCR (MSP)

Bisulphite modification was performed using a DNA modification kit (Intergen) according to the manufacturer's protocol. The modified DNA was used for MSP. The sequences of the primers are summarized in Table 1. PCR was carried out for 35 cycles after the first denaturation at 95 °C for 1 min (95 °C for 30 s, the annealing temperature listed in Table 1 for 30 s, and 72 °C for 30 s), followed by a final 4 min extension at 72 °C. DNA of the colo-205 cell line, which is known to show hypermethylation of the *p16*^{INK4A} gene promoter by the MSP method, was used as a positive control [36]. In addition, DNA from normal skeletal muscle was used as a negative control. Each PCR product (10 µl) was loaded directly onto 2% agarose gels, stained with ethidium bromide, and directly visualized under UV illumination.

Statistical analysis

Fisher's exact test was used to evaluate the association between two dichotomous variables. The survival

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curves were analysed by the Kaplan–Meier method and univariate and multivariate survival analyses were performed using the log-rank test or the Cox proportional hazards regression models. Stepwise selection of the variables was used to determine the best predictors. A p value of less than 0.05 was considered to indicate statistical significance.

Results

Clinical and histological findings

The clinicopathological data are summarized in Table 2. The age of the patients ranged from 7 to 95 years (average 58.4 years). Forty-two patients were male and 35 were female. Forty-eight patients were affected at extra-abdominal sites (23 in the thigh; five in the chest/abdominal wall; four in the buttock; three in the upper arm; two in the groin, lower leg, and back; and one each in the neck, scapular region, axilla, elbow, forearm, perineum, and sole). Twenty-nine patients had tumours situated in the abdomen (16 in the retroperitoneum, 11 in the mesentery, and two in the abdominal cavity). Tumour size was available for 53 cases. Forty-two tumours exceeded 5 cm in diameter, whereas 11 tumours measured less than 5 cm. The histological subtypes were as follows: 63 tumours were of the classic type; six were pleomorphic [37]; four were myxoid [38]; three were epithelioid; and one was inflammatory. The mitotic rate ranged from 1 to 48 per 10 HPFs (mean 12.8 per 10 HPFs). Forty-four cases had less than 10 mitoses per 10 HPFs, whereas 15 cases had between 10 and 20 mitoses per 10 HPFs, and 18 had more than 20 mitoses per 10 HPFs. According to the FNCLCC grading system, 29 tumours were grade 1; 33 were grade 2; and 15 were grade 3. The AJCC stage was evaluable in 53 cases. Twelve cases were considered to be AJCC stage I; 29 cases were stage II; six cases were stage III; and six cases were stage IV. In the univariate analysis, there was a statistically significant correlation between large tumour size and poor survival ($p = 0.025$).

Immunohistochemical analysis of p16

Decreased expression of p16 protein was identified in 25 of 77 LMS tumours (32%) (Figures 1A–1C). Of 25 tumours with decreased expression, eight showed complete loss of p16 expression and 17 cases showed

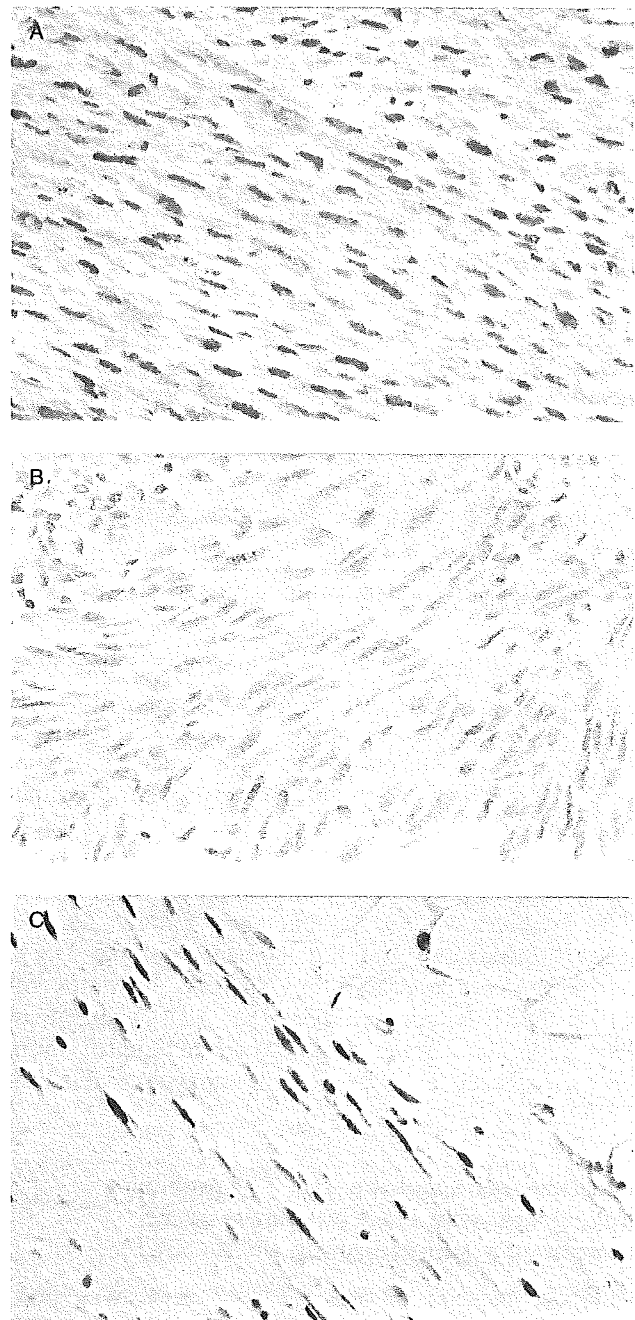

Figure 1. Immunohistochemical staining for p16. (A) Immunohistochemistry showing nuclear staining in the majority of tumour cells. This tumour showed neither homozygous deletion nor hypermethylation of the $p16^{INK4A}$ promoter (case L6) (×360). Loss of p16 expression was observed in the majority of tumour cells (B) (×360), but strong nuclear positivity was present in the surrounding normal tissue (C) (×360). This case showed hypermethylation of the $p16^{INK4A}$ promoter (case L10)

Table 2. Clinicopathological parameters in 77 cases of leiomyosarcoma

Age (years)		Sex		Location		Size (cm)		Mitotic rate (per 10 HPFs)		AJCC stage		FNCLCC grade	
≥60	34	Male	42	Extra-abdominal	48	≥5	42	≤9	44	I	12	Grade 1	29
<60	43	Female	35	Abdominal	29	<5	11	10–19	15	II	29	Grade 2	33
						Unknown	24	≥20	18	III	6	Grade 3	15
										IV	6		
										Unknown	24		

HPFs = high-power fields.

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Table 3. Correlation between p16 immunoreactivity and clinicopathological parameters in leiomyosarcoma

	p16 IHC		
	−	+	p value
Age, years			
≥60 (n = 34)	10	24	0.6339
<60 (n = 43)	15	28	
Sex			
Male (n = 42)	13	29	0.8099
Female (n = 35)	12	23	
Location			
Extra-abdominal (n = 48)	13	35	0.2176
Abdominal (n = 29)	12	17	
Size, cm			
≥5 (n = 42)	20	22	0.0038*
<5 (n = 11)	0	11	
Mitotic rate (per 10 HPFs)			
≥20 (n = 18)	8	10	0.2441
<20 (n = 59)	16	43	
AJCC stage			
I and II (n = 41)	17	24	0.4996
III and IV (n = 12)	3	9	
FNCLCC grade			
1 (n = 11)	18	29	0.4597
2 and 3 (n = 48)	14	34	

Fisher's exact test.
IHC = immunohistochemistry; HPFs = high-power fields.
* Statistically significant.

a focal loss. Twenty of 42 (48%) tumours larger than 5 cm showed decreased expression of p16 protein, whereas no decreased expression of p16 was observed in any of the tumours that measured less than 5 cm in diameter. There was a statistically significant correlation between decreased expression of p16 and large tumour size ($p = 0.0038$) (Table 3). With regard to the location of the tumours, 13 of 48 (27%) extra-abdominal-type LMSs showed decreased expression of p16 protein, whereas 12 of 29 (41%) abdominal-type LMSs revealed decreased expression. Decreased expression of p16 had no statistically significant correlation with tumour location. Univariate analysis revealed a statistically significant correlation between decreased expression of p16 protein and poor prognosis in the 60 cases for which survival information was available ($p = 0.0021$) and in the extra-abdominal-type LMSs ($p = 0.0014$), respectively (Figures 2A and 2B). However, decreased expression of p16 protein showed no correlation with prognosis in patients with abdominal-type LMS, nor was decreased p16 expression correlated with tumours of any group measuring 5 cm or less. Furthermore, no correlation was observed between p16 immunoreactivity and other clinicopathological parameters. Multivariate analysis of the 60 cases for which survival data were available revealed that p16 expression was the only independent prognostic factor for disease-specific survival (Table 4).

Table 4. Survival analysis in soft tissue leiomyosarcoma (n = 60)

	p value on survival analysis	
Variable	Univariate	Multivariate
Age (≥60 years)	0.5791	0.2423
Location (abdominal type)	0.3334	0.8634
Size (≥5 cm)	0.025*	0.3021
Mitotic rate (≥20)	0.5494	0.4477
AJCC stage (stages III and IV)	0.7546	0.1138
FNCLCC grade (grades 2 and 3)	0.2235	0.1544
Histological subtype (pleomorphic)	0.0751	0.195
p16 IHC (decreased expression)	0.0021*	0.012*

IHC = immunohistochemistry.
* Statistically significant.

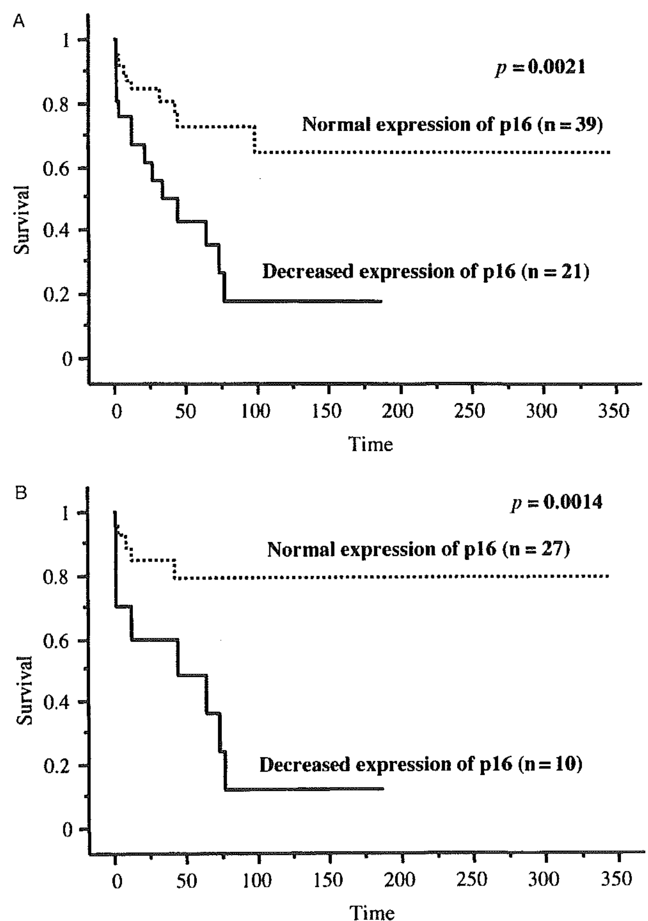

Figure 2. Effect on survival of decreased p16 expression. (A) Decreased p16 expression was associated with a significant reduction in overall survival ($p = 0.0021$). (B) Decreased p16 expression correlated significantly with poor survival in patients with extra-abdominal tumours ($p = 0.0014$)

Hypermethylation of the *p16^INK4A^* gene promoter

We next examined possible mechanisms of inactivation of the *p16^INK4A^* gene, because decreased expression of p16 protein was revealed by multivariate analysis to be the only independent prognostic factor amongst those considered here. First, we considered the methylation status of the *p16^INK4A^* gene. Analysis for hypermethylation of the *p16^INK4A^* gene

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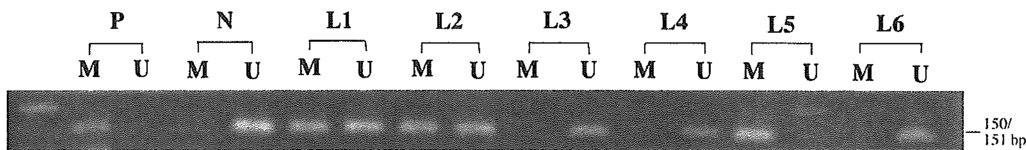

Figure 3. Methylation-specific PCR analysis of $p16^{INK4A}$. PCR products amplified using unmethylated (U) and methylated (M) specific primers. Cases L1, L2, and L5 show hypermethylation of the $p16^{INK4}$ gene promoter. Case L5 reveals only the methylated signal; however, the other two cases show both methylated and unmethylated signals. P = positive control; N = normal skeletal muscle

promoter was performed in 49 cases, ie in 15 cases with decreased p16 expression and in 34 cases without decreased p16 expression. Promoter hypermethylation was detected in 11 of 49 LMSs (22%). In 9 of 11 LMSs with promoter hypermethylation, both unmethylated and methylated signals were seen, whereas in two cases, only a methylated signal was observed (Figure 3). Those cases showing only the methylated signal revealed a diffuse decrease in p16 protein expression. Promoter hypermethylation was detected in 8 of 15 cases with decreased p16 protein expression and in 3 of 34 cases without decreased expression. There was a significant correlation between decreased expression of p16 protein and hypermethylation of the $p16^{INK4A}$ gene promoter ($p = 0.0014$) (Table 5). Furthermore, univariate analysis of 37 cases for which survival data were available showed that hypermethylation of the $p16^{INK4A}$ gene promoter had a statistically significant adverse effect on survival ($p = 0.0088$) (Figure 4). There was no statistically significant correlation between $p16^{INK4A}$ methylation status and the other clinicopathological parameters considered in this study (data not shown).

Homozygous deletion

Homozygous deletion of the $p16^{INK4A}$ gene was detected in 3 of 49 LMSs examined (6%) (Figure 5). All three tumours with homozygous deletion showed decreased expression of p16 protein. There was a statistically significant correlation between homozygous $p16^{INK4A}$ deletion and decreased p16 protein expression ($p = 0.0247$) (Table 6). Two patients with homozygous deletion of the $p16^{INK4A}$ gene died within 6 months and the remaining patient with this deletion died 34 months after the initial diagnosis.

Table 5. Correlation between p16 immunoreactivity and $p16^{INK4}$ methylation

	p16 IHC		
	−	+	*p* value
$p16^{INK4}$ methylation			
−	7	31	0.0014*
+	8	3	

Fisher's exact test.
IHC = immunohistochemistry.
* Statistically significant.

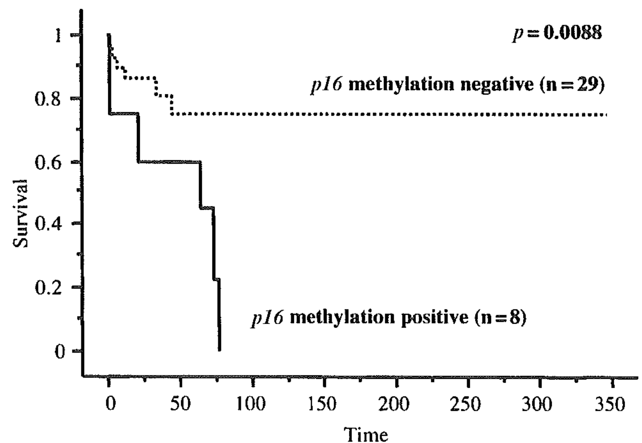

Figure 4. Effect of $p16^{INK4}$ promoter hypermethylation. Patients with methylation had a significantly poorer prognosis ($p = 0.0088$)

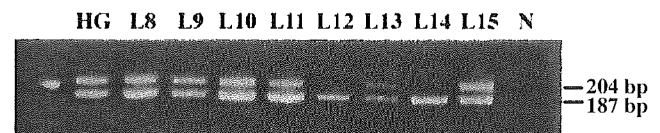

Figure 5. Differential PCR for homozygous deletion of $p16^{INK4}$. Cases L12 and L14 display very low levels (<25% ratio). HG = human genomic DNA; N = negative control.

Table 6. Correlation between p16 immunoreactivity and $p16^{INK4}$ homozygous deletion

	p16 IHC		
	−	+	*p* value
p16 HD			
−	12	34	0.0247*
+	3	0	

Fisher's exact test.
IHC = immunohistochemistry; HD = homozygous deletion.
* Statistically significant.

$p16^{INK4A}$ gene mutation

Mutation analysis of the $p16^{INK4A}$ gene was performed from exon 1 to exon 3, but mutation was not detected in any of the tumours analysed (Figure 6).

Discussion

The putative tumour suppressor gene $p16^{INK4a}$ encodes an important cyclin-dependent kinase (CDK)

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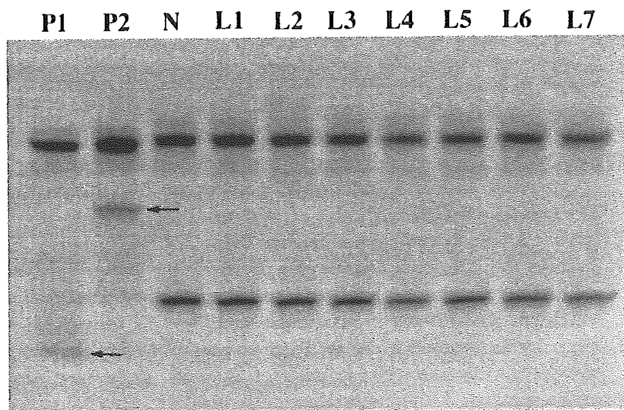

Figure 6. PCR–SSCP analysis of exon 1. Abnormal shifted bands were not evident in any of the LMS cases studied. P1, P2 = positive controls; N = negative control

inhibitor, which negatively regulates the G1–S transition of proliferating cells by contributing to the maintenance of pRb in an active state [1–3]. A decrease or total loss of p16 protein expression has been described in many types of carcinoma, as well as in a number of other types of malignancies [9,39]. Decreased expression of p16 protein has been detected in approximately 28% of various types of sarcoma; in particular, it was observed in 5–33% of LMSs [8,12,15,27,40]. Likewise, in our series, 32% of the LMSs displayed decreased expression of p16. In addition, decreased p16 protein expression correlates with tumour progression or with decreased survival among patients with carcinoma of the lung, pancreas, oesophagus, and malignant melanoma [4–7]. Similarly, Maitra *et al* reported that the absence of p16 expression correlated significantly with decreased survival in patients with paediatric osteosarcoma [8]. With regard to prognostic factors in LMS, the depth and size of tumours have been reported to be the most reliable indicators [40,41]. In addition, pleomorphic LMS has been demonstrated to have a more aggressive clinical course [37]. In addition, the AJCC stage is an important predictor of poor prognosis in patients with LMS [42]. This study, which included some cases in which the follow-up interval was short, showed that patients with advanced AJCC stage seemed to have a worse prognosis; however, this was not statistically significant. In our series, the patients with large tumours had a statistically significant unfavourable clinical course upon univariate analysis, which was consistent with previous findings. Furthermore, the presence of a relatively large tumour correlated closely with decreased expression of p16 overall. These findings indicate that p16 might function as a proliferative repressor in smaller-sized LMSs if the results are considered from the viewpoint of tumour growth. Furthermore, in the multivariate analysis of the 60 cases for which survival data were available, p16 expression was the only independent prognostic factor. Taking these findings into consideration, it is possible that the assessment of p16 expression might be useful in predicting the prognosis of patients with LMS.

The following primary mechanisms have been postulated for inactivation of the $p16^{INK4A}$ gene: homozygous deletion and promoter hypermethylation, with intragenic mutation occurring in only a small proportion of tumours. Homozygous deletion of the $p16^{INK4A}$ gene has been demonstrated in previous reports and is widely regarded as a major target associated with 9p21 deletion [11]. Inactivation of $p16^{INK4A}$ by homozygous deletion has also been reported in sarcomas and its frequency has been shown to range between 5% and 26% [12–14,28]. In the present study, homozygous deletion was detected in only 3 of 49 cases (6%). Previous studies have revealed that homozygous deletion correlates strongly with the inactivation of $p16^{INK4A}$ in malignancies, including sarcomas [12–14,24,43]. In our study, although uncommon, all cases with homozygous deletion were associated with silencing of $p16^{INK4A}$. On the other hand, mutations of $p16^{INK4A}$ have been detected infrequently in primary malignancies, including in sarcomas [25]. Dei Tos *et al* reported that a $p16^{INK4A}$ mutation was detected in one of 19 cases (5%) of LMS [15]. In the present study, no $p16^{INK4A}$ mutation was identified in any of the cases analysed. These results suggest that homozygous deletion and mutation of $p16^{INK4A}$ are rare inactivating events in LMS.

Methylation of the 5′ regulatory region, or of discrete regions of CG dinucleotides known as CpG islands, is an important mechanism of transcriptional repression [44]. Methylation of the 5′ CpG island of $p16^{INK4A}$ has been described in various types of cancer and the frequency of methylation varies according to the tumour type [43]. In sarcomas, the frequency of methylation of the $p16^{INK4A}$ promoter ranges from 0% to 35% [20–24,26]. In certain malignancies, some investigators have demonstrated that hypermethylation of the $p16^{INK4A}$ promoter correlates with the absence of p16 protein [16–18,45]. In previous studies of sarcomas, methylation of the $p16^{INK4A}$ gene was found in 50–57% of cases [20,21,26] involving patients with a lost or decreased p16 expression, indicating that promoter hypermethylation has a significant effect on the down-regulation of p16 protein. In the present study, methylation analysis by methylation-specific PCR showed $p16^{INK4A}$ promoter hypermethylation in 8 of 15 cases (53%) with a decrease or loss of p16 protein. Moreover, there was a close correlation between methylation status and the immunohistochemical findings. Taking these findings into consideration, we conclude that inactivation of the $p16^{INK4A}$ gene by promoter hypermethylation is a relatively frequent event in LMS of soft tissue. The most frequent mechanism for inactivation of the $p16^{INK4A}$ gene in our series was transcriptional repression due to DNA methylation at a CpG island in the promoter region, which was detected in half of the cases with decreased p16 expression. However, in 9 of 11 LMSs with promoter

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methylation, both unmethylated and methylated signals were seen by methylation-specific PCR. This may have been due to incomplete gene silencing resulting from histological heterogeneity or contamination with normal tissue. Three of 11 tumours with promoter methylation showed normal expression of p16 protein. However, if one considers Knudson's two-hit hypothesis [46], in which tumour suppressor gene function is lost by independent inactivation events involving both parental alleles, the present results might indicate that *p16*^{INK4A} methylation had occurred in only one allele in these cases.

Previous reports have demonstrated that the homozygous deletion of tumour suppressor genes plays an important role in the development and progression of some malignancies, as does hypermethylation [47,48]. Follow-up of the patients with tumours showing *p16*^{INK4A} methylation indicated that this gene is involved in the development of metastases in patients with melanoma [47]. Orlow *et al* reported statistically significant associations between an *INK4A/INK4B* deletion and poor prognosis in various types of sarcoma [22]. Some time later, other investigators confirmed these findings in patients with Ewing's sarcoma of bone [24,25]. In the present study, although two of three cases with the homozygous deletion had poor survival (both died within 6 months), our sample size was too small to confirm the association. On the other hand, we found a significant association with poor overall survival in the patients with hypermethylation of the *p16*^{INK4A} gene promoter. Although the participation of other prognostic factors should still be considered, our results suggest that hypermethylation of the *p16*^{INK4A} gene promoter may be a potential biomarker for risk prediction in patients with LMS of soft tissue.

In conclusion, we examined the immunohistochemical expression of p16 in a large series of soft tissue LMSs. Decreased expression of p16 was the only independent prognostic factor for poor survival among those studied here, as assessed by multivariate survival analysis that included the conventional clinicopathological parameters. In addition, we considered the mechanisms of *p16*^{INK4A} gene inactivation in patients with soft tissue LMS. Although homozygous deletion and mutation of *p16*^{INK4A} were rare events, hypermethylation of the *p16*^{INK4A} promoter was a frequent event that could account for p16 inactivation in approximately half of the tumours. The present results may eventually lead to the development of new therapeutic strategies, such as *p16*^{INK4A} gene therapy, for the treatment of patients with LMS.

Acknowledgements

We are grateful to Miss Y Nouzuka for her excellent technical assistance. This work was supported by a Grant-in-Aid for Scientific Research (C) (no. 15590304) from the Japan Society for the Promotion of Science, Tokyo, Japan.

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Diabetologia (2004) 47:1411–1415
DOI 10.1007/s00125-004-1466-8

Diabetologia

Comparison of diagnostic methods for diabetes mellitus based on prevalence of retinopathy in a Japanese population: the Hisayama Study

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Abstract

Aims/hypothesis. The aims of this study were to compare the ability of tests measuring fasting plasma glucose, 2-h plasma glucose and HbA_1c levels in predicting specific diabetic retinopathy, and to determine the cut-off level of each measurement for diagnosing diabetes in a Japanese population.
Methods. In a total of 1637 subjects, fasting plasma glucose, 2-h plasma glucose and HbA_1c levels were measured in a 75-g oral glucose tolerance test, and diabetic retinopathy was assessed by ophthalmic examination. We calculated receiver operating characteristic (ROC) curves as well as the prevalence of diabetic retinopathy by deciles of the distribution of these glycaemic measurements.
Results. Of the subjects, 37 (2.3%) had diabetic retinopathy. The prevalence of retinopathy dramatically increased in the tenth decile of each variable. Analysis with ROC curves showed that the optimal cut-off levels for diagnosis of diabetes were 6.4 mmol/l for fasting plasma glucose, 11.1 mmol/l for 2-h plasma glucose, and 5.7% for HbA_1c. The sensitivities for the cut-off point of the three measurements were identical (86.5%), and the specificities were similar (fasting plasma glucose 87.3%; 2-h plasma glucose 89.6%; HbA_1c 90.1%). The area under the ROC curve for 2-h plasma glucose (96.1%) was slightly but not significantly larger than that for fasting plasma glucose (90.0%) and that for HbA_1c (94.5%).
Conclusions/interpretation. Our findings suggest that measuring fasting plasma glucose or HbA_1c is just as useful as measuring 2-h plasma glucose for the diagnosis of diabetes, and that the cut-off point for diagnostic fasting plasma glucose level is lower than that of the current diagnostic criteria.

Keywords Diabetic retinopathy · Fasting plasma glucose · HbA_1c · Receiver operating characteristic curve

Received: 10 February 2004 / Accepted: 15 April 2004
Published online: 28 July 2004
© Springer-Verlag 2004

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Abbreviations: ADA, American Diabetes Association · FPG, fasting plasma glucose · 2-h PG, 2-hour post-load plasma glucose · NHANES III, the Third National Health and Nutrition Examination Survey · ROC, receiver operating characteristic

Introduction

In 1997 the Expert Committee of the American Diabetes Association (ADA) proposed new revised criteria for diagnosing diabetes [1]. These criteria lowered the diagnostic fasting plasma glucose (FPG) from ≥7.8 mmol/l to ≥7.0 mmol/l, but kept the 2-hour post-load plasma glucose (2-h PG) value at ≥11.1 mmol/l [1]. The WHO subsequently endorsed these recommendations [2]. These diagnostic levels were determined based on the findings of several population-based studies, including those of Pima Indians [3], the Third National Health and Nutrition Examination Survey (NHANES III) participants [1], and Egyptians [4]. However, the sensitivity and tolerability to glucose

load have been reported to vary among ethnic groups [5, 6, 7], and little information has been made available for the Japanese population [8]. In the present article, therefore, we compared the efficacy of tests for FPG, 2-h PG and HbA_1c levels in predicting specific diabetic retinopathy in a Japanese population, and determined the cut-off levels by separating the subjects who were at substantially increased risk of diabetic retinopathy from those who were not.

Subjects and methods

Study population. The Hisayama study is an ongoing prospective cohort study on cardiovascular disease and its risk factors in a community of Hisayama Town adjoining Fukuoka City, a metropolitan area in southern Japan. The enrolment criteria, characteristics of the study population, and overall design of this study have been described in detail in previous studies [9, 10, 11]. As part of the follow-up survey in 1998, we performed a cross-sectional examination, including a 75-g OGTT and ophthalmic examination, of Hisayama residents aged 40 to 79 years. Of a total of 3847 residents in that age group, 1950 subjects (50.7%) consented to participate in the study. After excluding 41 subjects who had already eaten breakfast at the examination, three who were on insulin therapy, 182 who underwent the examination at home, and 87 in whom gradable fundus photographs could not be obtained, a total of 1637 individuals (637 men and 1000 women) successfully completed the 75-g OGTT and ophthalmic examination.

Laboratory measurements. Blood samples were collected from an antecubital vein after an overnight fast for the determination of plasma glucose and HbA_1c levels. After the fasting blood specimen had been taken, the OGTT was performed with a 75-g glucose equivalent carbohydrate load (Trelan G; Shimizu Pharmaceutical, Shimizu, Japan) between 08.00 and 10.30 hours. Subjects receiving oral hypoglycaemic agents omitted their medication until the OGTT. At 120 min after ingestion of the solution, a blood sample was obtained for the determination of post-loading plasma glucose levels. These specimens were analysed within 24 h. Plasma glucose was determined by the glucose–oxidase method, and HbA_1c was measured by a high-pressure lipid chromatographic assay.

Ophthalmic examination and classification of diabetic retinopathy. Each participant underwent comprehensive ophthalmic examination, including stereoscopic fundus examination using indirect ophthalmoscopy, and examination with a slit lamp biomicroscope with a "superfield lens" (Volk, Mentor, Ohio, USA) after pupil dilatation. Fundus photographs (45°) were taken using a Topcon "non-mydriatic" TRC NW-5 fundus camera (Topcon, Tokyo, Japan) and Fujichrome slide film (Sensia II; Fujifilm, Tokyo, Japan). The presence of diabetic retinopathy was determined based on the grading of fundus examinations by indirect ophthalmoscopy, slit lamp, and colour fundus photographs. The photographs were graded by masked photo graders using a modification of the Airlie House classification system: (i) no retinopathic changes; (ii) mild non-proliferative retinopathy; (iii) moderate retinopathy; and (iv) proliferative retinopathy [12, 13, 14, 15]. The degree of diabetic retinopathy was determined according to the grading in the worse eye.

Statistical analysis. The SAS computer package (SAS Institute, Cary, N.C., USA) and Stata version 8.0 (Stata, College Station, Tex., USA) were used to perform all statistical analyses. The sensitivity of a specific glycaemic cut-off point was defined as its ability to correctly identify individuals who have diabetic retinopathy, and its specificity was defined as its ability to correctly identify individuals who do not have diabetic retinopathy. To compare the ability of FPG, 2-h PG and HbA_1c measurements to detect the presence or absence of retinopathy over a range of values, we calculated receiver operating characteristic (ROC) curves and compared the areas beneath them [16, 17]. The diagnostic properties of specific cut-off levels of FPG, 2-h PG and HbA_1c concentrations were defined by maximising the sensitivity and specificity to identify diabetic retinopathy. A two-sided p value of less than 0.05 was considered statistically significant.

Ethical considerations. This study was approved by the Human Ethics Review Committee of Kyushu University Graduate School of Medical Sciences, and was carried out in accordance with the Declaration of Helsinki. Informed consent was obtained from all participants.

Results

Of the study participants, 37 (2.3%) were found to have diabetic retinopathy. Mild non-proliferative retinopathy (category ii), moderate retinopathy (category iii) and proliferative retinopathy (category iv) were found in 27 (1.6%), 5 (0.3%) and 5 (0.3%) subjects respectively. When the subjects were divided according to the ADA fasting glucose criteria, diabetic retinopathy was found in four subjects (0.3%) with an FPG level of <6.1 mmol/l, in nine (5.9%) with an FPG of 6.1 to 6.9 mmol/l, and in 24 (23.1%) with an FPG of ≥7.0 mmol/l (Table 1). Likewise, five subjects (1.8%) with a 2-h PG level of 7.8 to 11.0 mmol/l and 32 (20.5%) with a 2-h PG of ≥11.1 mmol/l had some degree of retinopathy, but there was no subject with retinopathy in the group with a 2-h PG of <7.8 mmol/l.

Figure 1 shows the prevalence of diabetic retinopathy by deciles of the distribution of the FPG, 2-h PG and HbA_1c levels. All three measures of glycaemia were strongly associated with retinopathy, and the prevalence increased dramatically in the tenth decile of each variable, corresponding to an FPG of ≥6.5 mmol/l, a 2-h PG of ≥11.0 mmol/l, and HbA_1c levels of ≥5.8%. The prevalences of retinopathy in the tenth decile of the FPG, 2-h PG and HbA_1c levels were 16%, 20% and 20% respectively, while those in the ninth decile were 3%, 2% and 2% respectively.

To compare the ability of each glycaemic measurement to predict the presence of diabetic retinopathy, we calculated the sensitivity and specificity, and plotted ROC curves. As shown in Figure 2, the area under the ROC curve for 2-h PG was 96.1% (95% CI: 94.4–97.7) and was slightly but not significantly larger than that for FPG (90.0%; 95% CI: 83.8–96.7; p=0.076) and that for HbA_1c (94.5%; 95% CI: 91.6–97.5; p=0.296). The cut-off level defined by the

Table 1. The prevalence of diabetic retinopathy by fasting and 2-h plasma glucose levels defined by the 1997 ADA criteria, the Hisayama Study 1998

	Population at risk	Mild retinopathy*n* (%)	Moderate retinopathy*n* (%)	Proliferative retinopathy*n* (%)	Any retinopathy *n* (%)
FPG (mmol/l)					
<6.1	1383	4 (0.3)	0 (0.0)	0 (0.0)	4 (0.3)
6.1–6.9	152	9 (5.9)	0 (0.0)	0 (0.0)	9 (5.9)
≥7.0	104	14 (13.5)	5 (4.8)	5 (4.8)	24 (23.1)
2-h PG (mmol/l)					
<7.8	1201	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
7.8–11.0	280	5 (1.8)	0 (0.0)	0 (0.0)	5 (1.8)
≥11.1	156	22 (14.1)	5 (3.2)	5 (3.2)	32 (20.5)

FPG, fasting plasma glucose; 2-h PG, 2-hour plasma glucose

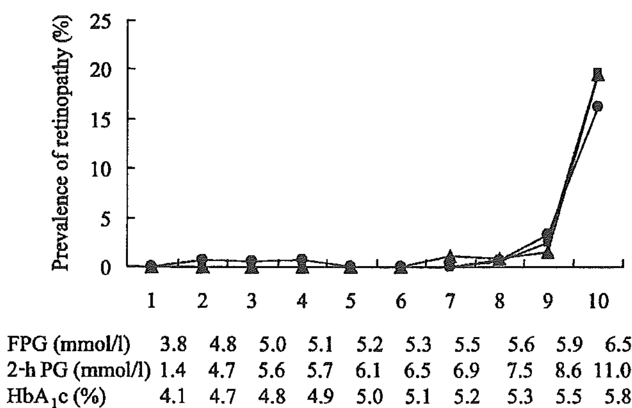

Fig. 1. Prevalence of retinopathy by deciles of the distribution of FPG, 2-h PG and HbA_1c levels in the Hisayama study. The *x* axis labels indicate the lower limit of each decile group. Black circles, FPG; black squares, 2-h PG; black triangles, HbA_1c

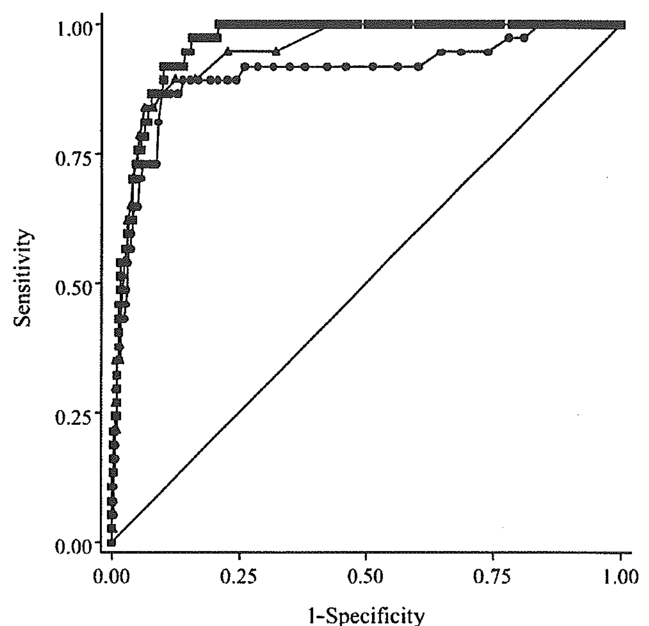

Fig. 2. Receiver operating characteristics (ROC) curves for FPG, 2-h PG and HbA_1c measures for predicting the presence of diabetic retinopathy. Black circles, FPG (ROC area 90.0%); black squares, 2-h PG (ROC area 96.1%); black triangles, HbA_1c (ROC area 94.5%); black line, reference

Table 2. Optimal cut-off points defined by maximising the sensitivity and specificity to identify diabetic retinopathy, the Hisayama Study 1998

	FPG	2-h PG	HbA_1c
Cut-off point	6.4 mmol/l	11.1 mmol/ l	5.7%
Sensitivity (%)	86.5	86.5	86.5
Specificity (%)	87.3	89.6	90.1
ROC curve area (%)	90.0	96.1	94.5

FPG, fasting plasma glucose; 2-h PG, 2-hour plasma glucose; ROC, receiver operating characteristic

maximum of sensitivity and specificity was 6.4 mmol/l for FPG, 11.1 mmol/l for 2-h PG, and 5.7% for HbA_1c (Table 2). The sensitivities of these cut-off points for the three measurements were identical (86.5%), and the specificities were similar (FPG 87.3%; 2-h PG 89.6%; HbA_1c 90.1%). The specificity for the FPG level of 7.0 mmol/l was higher (91.3%), but its sensitivity (70.3%) was lower than that for the FPG of 6.4 mmol/l.

Discussion

The current guidelines for the diagnosis of diabetes are based on several population studies examining the relationship between the measures of glycaemia and retinopathy [1]. The Hisayama study has allowed us to collect data on a large number of Japanese individuals with a range of glucose levels. We compared the efficacy of tests measuring FPG, 2-h PG and HbA_1c levels in predicting diabetic retinopathy, and found no significant difference in the ability to predict retinopathy among the three measures of glycaemia. In our subjects, the optimal cut-off levels defined by the ROC curves maximising the sensitivity and specificity

to identify diabetic retinopathy were 6.4 mmol/l for FPG, 11.1 mmol/l for 2-h PG, and 5.7% for HbA_1c. In addition, according to the prevalence of retinopathy examined by deciles of the distribution of the FPG, 2-h PG and HbA_1c levels, a threshold was evident between the ninth and tenth deciles of each variable, below which retinopathy was almost absent and above which a distinct increase in retinopathy was observed. The cut-off point between the ninth and tenth deciles of each measure of glycaemia was very similar to that determined by the ROC curve. In contrast, the cut-off level of 2-h PG was consistent with the current ADA and WHO criteria. However, the cut-off level of FPG was slightly lower than that of the diagnostic criteria.

All three measures of glycaemia (FPG, 2-h PG and HbA_1c) were strongly associated with retinopathy in our Japanese population, which was similar to the findings in Pima Indians [3] and Egyptians [4], and to the results of the NHANES III [1]. The Egyptian study [4] reported that the area under the ROC curve for 2-h PG was similar to that for FPG, but that the area under the curve for HbA_1c was significantly smaller than that for FPG and 2-h PG. The authors concluded that the FPG and 2-h PG were each strongly and equally associated with retinopathy for diagnostic purposes. In contrast, in the Pima Indian study [3], ROC analysis showed that the area under the curve for 2-h PG was slightly but not significantly larger than that for FPG and that for HbA_1c. This result is consistent with that of the present study. Our findings, together with those of the Pima Indian study, suggest that all measures are equally effective for diagnostic purposes, and that the FPG or HbA_1c alone are also acceptable alternatives to 2-h PG, which is complicated to measure by OGTT.

Optimal cut-off levels of plasma glucose for defining diabetes vary between populations. In the Pima Indian study [3], the ROC curve analysis identified the optimal FPG cut-off level as 6.8 mmol/l. The NHANES III [1] also reported that the FPG cut-off level equivalent to the 2-h PG criterion of 11.1 mmol/l was 6.7 mmol/l. In contrast, we found that the optimal cut-off level of 2-h PG for diagnosis of diabetes, 11.1 mmol/l, was consistent with current ADA and WHO criteria. However, the optimal cut-off level of FPG, 6.4 mmol/l, was slightly lower than that of the diagnostic criteria [18]. Other Asian population studies [19, 20] have also reported that the optimal cut-off levels of FPG for diagnosis of diabetes ranged from 5.6 to 6.0 mmol/l in the sensitivity and specificity analysis for retinopathy. These findings suggest that the optimal level of diagnostic FPG is lower in Asian populations, including the Japanese population, than in western populations. In addition to the lower incidence of obesity in Asian populations, racial, genetic or environmental factors could contribute to this discrepancy.

This study has several limitations. Firstly, our results could be biased by the low participation rate. To ascertain the possibility of this bias, we compared the mean values of age, FPG, 2-h PG and HbA_1c levels as well as the proportion of men and women between the subjects who did participate in ophthalmic examination and those who did not. However, no significant differences in these parameters were observed between the groups, suggesting that this limitation does not invalidate the findings of the present study to a large extent. Secondly, our study population included individuals with diabetes who were taking oral anti-hyperglycaemic agents, resulting in a possible bias in the distribution of glycaemia. Although we performed analyses in which we excluded subjects on anti-hyperglycaemic medication, we could not draw any definitive conclusions due to the relatively low number of diabetic subjects not on anti-hyperglycaemic medication. However, it is to be expected that anti-hyperglycaemic medication has the same impact on FPG, 2-h PG and HbA_1c levels, and thus medication was unlikely to have affected our findings on the ability of these measurements to predict diabetic retinopathy. In addition, we computed the FPG level equivalent of the 2-h PG criterion of 11.1 mmol/l among Hisayama residents who underwent OGTT in 1988 and found it to be 6.2 mmol/l (data not shown). This value was similar to the optimal FPG cut-off level of 6.4 mmol/l in the present study. It was therefore suggested that this type of bias did not distort the conclusions of our study. Thirdly, because of the cross-sectional design of this study, it is still unclear how the onset of retinopathy is related to the three measures of glycaemia. Further prospective investigation would help to clarify this issue.

In conclusion, after consideration of the risk of diabetic retinopathy, our population-based study suggests that measuring FPG or HbA_1c is just as useful as measuring 2-h PG for the diagnosis of diabetes. Furthermore, there is a possibility that diabetic retinopathy occurs in patients with FPG levels lower than those currently used for the diagnosis of diabetes in the Japanese population.

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Relationship Between Plasma Glutathione Levels and Cardiovascular Disease in a Defined Population

The Hisayama Study

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Background and Purpose—Glutathione (GSH) appears to have marked antioxidant activities and therefore may prevent cardiovascular disease (CVD). However, there are very few reports on this subject. In a community-based case–control study, we tested the hypothesis that low levels of plasma GSH are closely associated with CVD and its clinical types.

Methods—The association between fasting plasma total GSH (tGSH) levels and CVD were assessed using conditional logistic regression analysis among 134 CVD cases and 435 age- and sex-matched healthy control subjects.

Results—Mean tGSH concentrations were lower in all CVD cases than in the control subjects (3.06 versus 3.71 μmol/L; $P=0.0001$). Among the CVD types, both the cerebral infarction cases (2.98 versus 3.59 μmol/L; $P=0.001$) and cerebral hemorrhage cases (2.51 versus 3.43 μmol/L; $P=0.0027$) had significantly lower tGSH levels than the corresponding control groups had. The same tendency was observed for cases of subarachnoid hemorrhage (3.45 versus 3.83 μmol/L; $P=0.36$) and myocardial infarction (3.65 versus 3.77 μmol/L; $P=0.69$), but these differences were not statistically significant. After adjustment for other confounding factors, the risk of CVD was significantly lower in the third (adjusted odds ratio, 041; 95% CI, 0.21 to 0.77) and the fourth quartiles (adjusted odds ratio, 0.25; 95% CI, 0.12 to 0.51) than in the first. This association was most prominent in patients with lacunar infarction or cerebral hemorrhage.

Conclusions—These findings suggest that reduced plasma tGSH levels are a risk factor for CVD, especially for cerebral small vessel disease. (***Stroke*. 2004;35:2072-2077.**)

Key Words: cardiovascular diseases ■ cerebral hemorrhage ■ lacunar infarction ■ risk factors

Oxidative stress appears to play a major role in the development of cardiovascular disease (CVD).[1] Several endogenous substances, including homocysteine, which may be involved in the production of oxygen radicals in vessel walls, are reported to promote atherosclerotic disease by causing oxidative vascular injury.[2] Conversely, antioxidants such as vitamin C, vitamin E, and carotene may have protective effects against the development of CVD.[3]

Glutathione (GSH), a sulfhydryl (SH)-containing tripeptide, has several major physiological functions: it maintains SH groups of proteins in a reduced state, participates in amino acid transport, detoxifies foreign compounds, enzymatically degenerates endogenous peroxides, forms bioactive molecules, and acts as a coenzyme in several enzymatic reactions.[2] GSH has also been demonstrated to play a role in detoxifying oxygen radicals and therefore may prevent cellular damage from oxidative stress.[2] Several clinical case–control studies have shown that patients under chronic disease states such as heart disease,[4] arthritis,[4,5] diabetes,[4,5] and malignancies[6] have lower plasma levels of GSH than control subjects, suggesting that GSH has a protective role against such diseases. As for CVD, only a few studies have associated GSH levels in plasma or red blood cells with coronary heart disease.[7,8] Thus far, no study has shown an association with stroke.

Since 1961, we have been performing a cohort study of CVD in the town of Hisayama, a suburban community of ≈7500 residents on Kyushu Island in Japan. The present report describes this population-based retrospective case–control study, which was designed to investigate the relationship between plasma total GSH (tGSH) levels and clinical types of CVD (namely, type-specific stroke and myocardial infarction) in the community of Hisayama.

Subjects and Methods

Patients and Control Subjects

Throughout the course of the Hisayama study, information concerning newly developed cases of CVD among residents was collected through weekly visits to local practitioners and major hospitals in

Received February 5, 2004; final revision received June 4, 2004; accepted June 22, 2004.

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***Stroke* is available at http://www.strokeaha.org** **DOI: 10.1161/01.STR.0000138022.86509.2d**

and around town.[9] Regular health checks were performed biennially to residents aged 40 years or older to obtain information about any new cardiovascular events missed by the monitoring network. Whenever a new cardiovascular event was suspected, one of the study physicians neurologically and physically examined the subject (ultimately including the majority of subjects) and collected clinical information, including that regarding the course of the disease, as soon as possible.

Stroke was defined as a sudden onset of nonconvulsive and focal neurological deficit persisting for >24 hours and was classified as cerebral infarction, cerebral hemorrhage, subarachnoid hemorrhage, or an undetermined type.[10] Morphological examinations by several imaging techniques or autopsy, or both, were performed on almost all stroke cases encountered.[11] Cerebral infarction was further subdivided into 4 clinical categories: lacunar infarction, atherothrombotic infarction, cardioembolic infarction, and undetermined subtypes, according to the criteria established previously and described in detail elsewhere.[11]

Diagnosis of myocardial infarction was based on detailed clinical information and at least one of the following findings: electrocardiographic evidence of myocardial infarction; elevated cardiac enzymes; or a morphological finding including echocardiographic, scintigraphic, and angiographic abnormalities compatible with myocardial injury.

From June to October 1996, we enrolled all of the town's prevalent cases of CVD, for a preliminary total of 176 patients with a history of stroke or myocardial infarction.[12] Excluding cases with severe disability or with undetermined stroke type, a total of 134 cases (69 men and 65 women; mean age, 72.0±4.6 years; range, 46 to 91 years) were eligible for the present study. The mean interval from the onset of CVD to blood sampling for plasma tGSH measurement was 7.5 years (range, 3 months to 30 years). The patient group included 75 cases of cerebral infarction, 28 cases of cerebral hemorrhage, 14 cases of subarachnoid hemorrhage, 21 cases of myocardial infarction, and 4 cases of simultaneous cerebral and myocardial infarctions. The 75 cerebral infarction cases were subdivided into 43 cases of lacunar infarction, 24 of atherothrombotic infarction, and 8 of cardioembolic infarction.

As a control group, Hisayama residents who were healthy and free from both stroke and myocardial infarction, and who had participated in the 1996 health checkup, were randomly selected. For each CVD case, there were 1 to 5 sex- and age-matched (±2 years) controls. The control group consisted of 435 individuals (246 men and 189 women; mean age, 67.9±2.4 years; range, 46 to 91 years).

Laboratory Measurement

During the screening period in 1996, blood samples were obtained from all cases and control subjects in an overnight fasting state. Plasma tGSH and total homocysteine levels in the collected samples of CVD cases and controls were measured, using the high-performance liquid chromatography method described previously by Toyo'oka et al[13] at the Saga Research Institute of Ohtsuka Pharmaceutical Co, Ltd, with no awareness of the case–control status or of clinical information. Plasma vitamin B_6 concentrations were also determined using high-performance liquid chromatography with fluorescence detection. A chemiluminescent immunoassay was used to measure plasma folate and vitamin B_{12}. Serum cholesterol levels were measured enzymatically, and total protein levels were determined by the Biuret method. Diabetes mellitus was determined by either a 75-g oral glucose tolerance test (the 1998 WHO criteria), casual blood glucose levels (>11.1 mmol/L), or a medical history of diabetes. Height and weight were measured in light clothes without shoes, and the body mass index (kg/m^2) was calculated. Sitting blood pressure was measured 3 times on the right upper arm using a sphygmomanometer after a rest of at least 5 minutes. The average of the 3 measurements was used for the analysis. Hypertension was defined as a systolic blood pressure reading ≥140 mm Hg, a diastolic blood pressure reading ≥90 mm Hg, or the current use of antihypertensive drugs. Questions on personal smoking habits and alcohol consumption were asked, and the subjects were categorized as either current users or not.

TABLE 1. Clinical Characteristics of the Study Subjects

Factors	Cases (n=134)	Controls (n=435)
Age, y	72±9*	68±9
Sex, % male	51†	57
Systolic blood pressure, mm Hg	145±3†	136±1
Diastolic blood pressure, mm Hg	80±2	77±1
Hypertension, %	56†	50
Diabetes, %	11†	9
Body mass index, kg/m^2	21.3±0.3†	22.2±0.2
Cholesterol, mmol/L	5.0±0.08*	5.3±0.04
Total protein, g/L	71±0.4*	72±0.2
Folate, nmol/L	6.1±0.3	6.6±0.2
Vitamin B_6, nmol/L	64.6±2.1*	83.8±1.5
Vitamin B_{12}, pmol/L	754±27†	666±13
Total homocysteine, μmol/L	12.8±0.3*	11.1±0.2
Drinking, %	30†	36
Smoking, %	21	25

All variables except for age and sex were adjusted for age and sex. Values are expressed as means±SE (for age, SD) and percentages. *$P<0.01$, †$P<0.05$ vs controls.

Statistical Analysis

The mean age was compared using the Student *t* test, as was the frequency of male gender using the χ^2 test. Age- and sex-adjusted mean values of relevant factors were calculated using the covariance method. Differences in the parameters between CVD cases and controls were assessed by the Student t test, and trends in the parameters among the tGSH quartiles were assessed by multiple linear regression analysis. The age- and sex-adjusted frequencies were calculated by the direct method, then compared by the Cochran–Mantel–Haenszel χ^2 test using 10-year age groupings with the total subjects as a standard.

The odds ratio (OR) and 95% CI of CVD and its clinical types were calculated by the distribution of tGSH tertiles or quartiles using conditional logistic regression analysis. A value of $P<0.05$ was considered statistically significant.

Ethical considerations

This study was conducted with the approval of the Human Ethics Review Committee of the Kyushu University Graduate School of Medical Sciences. Written informed consent for medical research was obtained from all participants.

Results

The clinical characteristics of CVD cases and control subjects are demonstrated in Table 1. Because there were fewer control subjects in the elderly than in the younger case–control sets, especially in the case of females, the mean age and proportion of women were higher in the CVD group than in the control group. Thus, comparisons for other variables were performed after adjusting for age and sex. Mean systolic blood pressure and the frequency of hypertension and diabetes were significantly higher among CVD cases than among control subjects. CVD patients had lower body mass index, serum cholesterol, and total protein levels. Although the plasma folate concentration was the same between CVD patients and controls, the former presented lower plasma vitamin B_6 and higher vitamin B_{12} levels than the latter. The mean total homocysteine levels were significantly higher in

TABLE 2. Comparison of Age- and Sex-Adjusted Mean Values±SE of Fasting Total Plasma Glutathione Concentrations Between Cases With Cardiovascular Disease and Controls

	Cases and Controls	Plasma Glutathione (μmol/L)	*P*
Cardiovascular disease	Case (n=134)	3.06±0.12	
	Control (n=435)	3.71±0.06	0.0001
Cerebral infarction	Case (n=75)	2.98±0.16	
	Control (n=248)	3.59±0.08	0.001
Cerebral hemorrhage	Case (n=28)	2.51±0.27	
	Control (n=121)	3.43±0.13	0.0027
Subarachnoid hemorrhage	Case (n=14)	3.45±0.37	
	Control (n=67)	3.83±0.17	0.36
Myocardial infarction	Case (n=21)	3.65±0.29	
	Control (n=95)	3.77±0.13	0.69

CVD cases than in the control subjects. Alcohol consumption was significantly less frequent in CVD patients than in the control subjects, whereas the frequency of smoking habits was the same between the 2 groups.

The age- and sex-adjusted mean values of plasma tGSH levels were significantly lower among CVD cases overall than among the control subjects (Table 2). Among CVD types, cases of cerebral infarction or hemorrhage had significantly lower tGSH levels than those of the respective corresponding control groups. A similar tendency was observed in cases of subarachnoid hemorrhage or myocardial infarction, although the differences were not statistically significant.

CVD patients and control subjects were combined into 1 group, then divided into quartiles based on their tGSH levels. The mean value or frequency of each relevant factor was then compared among the 4 groups (Table 3). Individuals who were included in the fourth quartile of tGSH were younger, but the proportion of men did not differ among the quartiles. The levels of systolic and diastolic blood pressures decreased with increasing tGSH levels, whereas the frequency of hypertension did not significantly differ among the 4 groups. The frequency of diabetes significantly decreased with elevating tGSH levels. Although the body mass index was the same across tGSH levels, serum cholesterol levels significantly increased with elevating tGSH. Individuals who were included in the first quartile of tGSH had low mean serum total protein and vitamin B_6 levels, whereas plasma folate and vitamin B_{12} levels were the same across all tGSH levels. There was no correlation between tGSH and total homocysteine levels. The frequency of alcohol consumption significantly decreased with increasing tGSH levels, although no such trend was seen in the frequency of smoking habits.

To further evaluate the association of CVD with tGSH levels, crude and multivariate-adjusted ORs were calculated by quartiles of tGSH levels (Table 4). Compared with the first quartile, in the third and fourth quartiles the risk of CVD decreased with elevating tGSH and was significantly lower in the third (crude OR, 0.41; 95% CI, 0.23 to 0.72) and the fourth (crude OR, 0.24; 95% CI, 0.12 to 0.46) quartiles. A similar pattern was observed for cerebral infarction and cerebral hemorrhage, but not for subarachnoid hemorrhage or myocardial infarction. The magnitude of the effect of tGSH on each type of CVD, except for cerebral hemorrhage in the fourth quartile, was not found to be attenuated substantially from quartile to quartile, even after adjustment for other confounding factors such as systolic blood pressure, diabetes, body mass index, cholesterol, total protein, folate, vitamin B_6, vitamin B_{12}, total homocysteine, smoking habits, and alcohol consumption.

TABLE 3. Age- and Sex-Adjusted Mean Values or Frequencies of Cardiovascular Risk Factors According to Quartiles of Total Glutathione Levels

	Quartiles of Glutathione (μmol/L)				
Factors	<2.53 (n=142)	2.53–3.41 (n=143)	3.41–4.4 (n=143)	>4.4 (n=141)	*P* for trend
Age, y	70±9	69±9	70±9	67±9	0.02
Sex, % male	61	52	53	56	0.39
Systolic blood pressure, mm Hg	142±2	141±2	135±2	133±2	0.0001
Diastolic blood pressure, mm Hg	78±1	79±1	77±1	74±1	0.0001
Hypertension, %	54	62	47	49	0.07
Diabetes, %	15	8	8	6	0.02
Body mass index, kg/m^2	22.0±0.3	22.4±0.3	22.3±0.3	21.4±0.3	0.13
Cholesterol, mmol/L	5.1±0.1	5.2±0.1	5.3±0.1	5.4±0.1	0.004
Total protein, g/L	70±0.4	72±0.4	73±0.4	72±0.4	0.0004
Folate, nmol/L	6.4±0.3	6.4±0.3	6.5±0.3	6.5±0.3	0.64
Vitamin B_6, nmol/L	68.2±2.2	74.1±2.2	92.9±2.2	82.3±2.2	0.008
Vitamin B_{12}, pmol/L	728±26	649±23	674±24	692±24	0.55
Total homocysteine, μmol/L	11.7±0.4	11.4±0.4	11.7±0.4	11.0±0.4	0.12
Drinking, %	41	34	29	29	0.008
Smoking, %	27	19	25	24	0.59

Age and sex were not age- and sex-adjusted. Values are expressed as means±SE (for age, SD) and percentages.

TABLE 4. Crude and Adjusted Odds Ratios of Cardiovascular Disease and its Types in Each Quartile of Total Glutathione Distribution

		Quartiles of Glutathione (μmol/L)				
		<2.53 OR	2.53–3.41 OR (95% CI)	3.41–4.4 OR (95% CI)	>4.4 OR (95% CI)	*P* for trend
		n=142	n=143	n=143	n=141	
Cardiovascular disease	Crude	1.0	0.54 (0.31–0.92)	0.41 (0.23–0.72)	0.24 (0.12–0.46)	0.0001
	Adjusted*	1.0	0.57 (0.31–1.05)	0.41 (0.21–0.77)	0.25 (0.12–0.51)	0.0001
		n=89	n=85	n=78	n=71	
Cerebral infarction	Crude	1.0	0.59 (0.29–1.2)	0.31 (0.14–0.68)	0.22 (0.15–0.32)	0.0001
	Adjusted*	1.0	0.55 (0.24–1.25)	0.29 (0.12–0.69)	0.19 (0.07–0.52)	0.0002
		n=46	n=45	n=29	n=29	
Cerebral hemorrhage	Crude	1.0	0.36 (0.13–1.02)	0.08 (0.01–0.63)†	0.24 (0.07–0.90)	0.006
	Adjusted*	1.0	0.37 (0.10–1.30)	0.05 (0.01–0.58)†	0.37 (0.08–1.69)	0.06
		n=17	n=12	n=28	n=24	
Subarachnoid hemorrhage	Crude	1.0	0.97 (0.14–6.88)	1.26 (0.29–5.55)	0.69 (0.13–3.82)	0.77
	Adjusted*	1.0	0.97 (0.14–6.88)	1.26 (0.29–5.55)	0.69 (0.13–3.82)	0.77
		n=22	n=28	n=33	n=33	
Myocardial infarction	Crude	1.0	1.97 (0.43–8.94)	1.81 (0.42–7.85)	0.45 (0.06–3.39)	0.52
	Adjusted*	1.0	3.51 (0.60–20.5)	2.39 (0.43–13.4)	0.43 (0.04–4.09)	0.40

OR indicates odds ratio.

*Adjusted for age, sex, systolic blood pressure, diabetes, body mass index, cholesterol, total protein, folate, vitamin B_6, vitamin B_{12}, total homocysteine, smoking, and drinking.

We further divided the combined group of patients with cerebral infarction and the corresponding control subjects into tertiles by tGSH levels and estimated the OR of each subtype of cerebral infarction (Table 5). The risk of lacunar infarction was significantly lower in the second and third tertiles than in the first. In the case of atherothrombotic infarction or cardioembolic infarction, however, the risk decreased with elevating tGSH levels. However, these trends were not statistically significant. Because there were no cases of cardioembolic infarction in the second tertile, we could not estimate OR for this tGSH level.

Discussion

The major new finding of the present study is that CVD cases had much lower levels of plasma tGSH than control subjects did. The risk of CVD continuously decreased with increasing

TABLE 5. Crude and Adjusted Odds Ratios of Subtypes of Cerebral Infarction in Each Tertile of Total Glutathione Distribution

		Tertile of Glutathione (μmol/L)			
Subtype of Cerebral Infarction		<2.9 OR	2.9–4.1 OR (95% CI)	>4.1 OR (95% CI)	*P* for trend
		n=68	n=66	n=69	
Lacunar infarction	Crude	1.0	0.35 (0.14–0.86)	0.33 (0.14–0.76)	0.009
	Adjusted*	1.0	0.22 (0.07–0.66)	0.23 (0.09–0.65)	0.02
		n=43	n=43	n=37	
Atherothrombotic infarction	Crude	1.0	0.49 (0.16–1.52)	0.46 (0.15–1.38)	0.15
	Adjusted*	1.0	0.45 (0.14–1.48)	0.47 (0.14–1.59)	0.21
		n=15	n=7	n=9	
Cardioembolic infarction	Crude	1.0	NA	0.30 (0.03–2.8)	0.25
	Adjusted	1.0	NA	0.30 (0.03–2.8)	0.25

OR indicates odds ratio; NA, not available.

*Adjusted for age, sex, systolic blood pressure, diabetes, body mass index, cholesterol, total protein, folate, vitamin B_6, vitamin B_{12}, total homocysteine, smoking, and drinking.

tGSH levels and was not attenuated even after adjustment for other confounding factors. Thus, the reduced level of plasma tGSH may be an independent risk factor for the development of CVD.

Among the clinical types of CVD, the risk of lacunar infarction and cerebral hemorrhage significantly decreased with elevating tertiles of tGSH. A similar tendency was observed for atherothrombotic, cardioembolic, and myocardial infarctions, although for these groups the difference was not statistically significant. It is well-known that arteriosclerotic lesions of the perforating intracerebral arteries induced mainly by chronic arterial hypertension contribute to the development of lacunar infarction and cerebral hemorrhage. However, both atherothrombotic infarction and myocardial infarction are the consequences of atherosclerosis of large cerebral and coronary arteries, and rupture of an intracranial saccular aneurysm is the most common cause of subarachnoid hemorrhage. γ-glutamyl transpeptidase, produced in the first step of the breakdown of GSH, is contained in larger quantities with much higher enzyme activity in the endothelium of capillaries than in that of larger vessels in the brain.[14] This suggests that the concentration of GSH in the brain is apt to decrease more in capillaries than in large arteries; consequently, cerebral small arteries may be more sensitive to fluctuation in levels of plasma GSH. However, atherothrombotic and myocardial infarctions are associated with major risk factors—such as hypertension, diabetes, and smoking—that carry greater exposure to oxidative stresses and therefore may be associated with tGSH deficiency. In addition, the sample size of atherothrombotic, cardioembolic, and myocardial infarction was insufficient to draw a conclusion. Thus, our findings imply that plasma tGSH offers a strong defense mechanism at least against arteriosclerosis of small cerebral arteries, whereas its preventive effects on atherosclerosis of large vessels are inconclusive.

Several mechanisms by which GSH may prevent cerebrovascular damage have been suggested. Harlan et al[15] showed that depletion of GSH by buthionine sulfoximine, an inhibitor of glutathione synthesis, augmented the endothelial damage caused by hydrogen peroxide released from activated neutrophils. Thus, GSH may have marked protective effects against oxidative damage by means of its direct antioxidative effects.[2] GSH has been reported also to play a role in the maintenance of SH groups and other cellular antioxidants in a reduced state, thereby maintaining their antioxidant effects.[2] In addition, Thomas et al[16] showed that both GSH and GSH-dependent selenoperoxidase protect cells against the damage induced by oxidized low-density lipoprotein. Presumably, this protection may be the result of detoxification of lipid hydroperoxides and the reduced formation of free radical intermediates with greater reactivity.[16]

Several limitations of our study should be discussed. The primary limitation is that our data were derived from a retrospective case–control study. Thus, we cannot exclude the possibility that decreased tGSH was a consequence of CVD or related conditions. Vegetarians were reported to have higher plasma levels of tGSH than nonvegetarians,[17] and healthy men receiving ascorbic acid-deficient diets had lower plasma tGSH levels than control subjects.[18] Thus, it is possible that changes in lifestyle after CVD onset, such as decreased dietary intake of vegetables and vitamins, may be related to or contribute to the decreased plasma tGSH levels in our patients. We did not examine dietary intake in this case–control study. However, the plasma concentrations of vitamin B_{12} and folate in our CVD patients were higher than or approximately equal to those of the controls, suggesting that the CVD patients did not have vitamin-deficient diets. The secondary limitation is that our study lacked information on drug use, which could affect plasma tGSH levels. Although the effects of drug use on tGSH levels have been scarcely studied, it has been reported that antihypertensive agents, long-acting nitrates, and aspirin, which are frequently used in CVD patients, did not affect plasma tGSH levels.[7] Thus, a bias from this source is unlikely. The third limitation is that our sample size of CVD patients is relatively small for subtype analysis, especially for myocardial infarction and the subtypes of stroke. Further study with a larger sample size is needed to establish more definitive conclusions.

In conclusion, a reduced level of tGSH may be an important risk factor for the development of CVD, and especially of lacunar infarction and cerebral hemorrhage. There is evidence that orally administered GSH increases its plasma concentrations in animals and humans.[19] Thus, it is anticipated that oral administration of GSH is a possible therapeutic strategy for the prevention of CVD, although further studies, including randomized, double-blind, and placebo-controlled trials, are essential to confirm the preventive effects of GSH against CVD.

Acknowledgments

The authors thank the residents of Hisayama Town, for their participation in the survey, and the staff of the Division of Health and Welfare of Hisayama Town, for their cooperation in this study. This study was supported in part by a grant-in-aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

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