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Osaka University Hospital, Osaka, Japan

Osaka University Hospital started from 1869 as a state-run hospital and has been playing a central role in medical care in western Japan. A number of historic treatments, *e.g.* combined heart and lung transplantation (performed in 2009, the first registry report in Japan), have been accomplished. Currently, this hospital is the only one institute in Japan that is approved for transplantation of all transplantable organs from brain dead donors (heart, lung, combined heart and lung, liver, pancreas, and small intestine).

(Photo by Yoshinori Inagaki)

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Review

Metabolic syndrome: What are the risks for humans?

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Summary **Metabolic syndrome (MetS) is a widely prevalent and multi-factorial disorder. The syndrome has been given several names such as insulin resistance (IR) syndrome, plurimetabolic syndrome, Reaven's syndrome, Syndrome X, and the deadly quartet. The formulation of National Cholesterol Education Program Adult Treatment Panel III (NCEP-ATP) guidelines has led to some uniformity and standardization of the definition of MetS and has been helpful epidemiologically. The clinical relevance of MetS is related to its role in the development of cardiovascular disease. Weight reduction is one of the mainstays of treatment. This article provides a comprehensive discussion of metabolic risk factors, the history of MetS, and its diagnosis, epidemiology, etiology, pathophysiology, and treatment. There is a need to comprehensively review this particular syndrome in view of the ever increasing-incidence of this condition.**

Keywords: Metabolic syndrome (MetS), human, health

1. Introduction

India is a major contributor to the global increase in cardiovascular disease through the increased mortality and prevalence of metabolic syndrome (MetS). MetS is a constellation of physiological and biochemical abnormalities characterized by diabetes or high fasting glucose, central obesity, abnormal cholesterol and triglyceride (TG) levels, and hypertension (*1*,*2*). This clustering of abnormalities is frequently seen and attributed to people's dietary habits. One in approximately every 4 or 5 adults has developed MetS depending on the environmental conditions and daily lifestyle habits of the country where he or she resides. The incidence of this syndrome has been estimated to increase with age for individuals over 50 years of age. MetS affects 27% of the population in India, nearly 30% in Europe (*2*), and more than 40% in the US (*3*). MetS has been accepted worldwide as a clinical marker for earlier detection of cardiovascular disease and type 2 diabetes (*4*,*5*). People with MetS are estimated to have twice the risk of developing cardiovascular disease compared to healthy individuals and a five-fold increased risk of type 2 diabetes (*1*,*5*). However, the underlying pathophysiological processes leading to its development are unclear and there is confusion over its conceptual definitions and criteria, allowing the medical controversy over MetS to continue. An increase in total body fatness and preferential upper body accumulation of fat is independently related to insulin resistance (IR). Obese women with a greater proportion of upper body fat tend to be more insulin-resistant, hyperinsulinemic, glucose-intolerant, and dyslipidemic than obese women with a greater proportion of lower body fat. Therefore, the distribution of body fat is an important correlate of MetS. The term "metabolic" refers to the biochemical processes involved in the body's normal functioning. Risk factors are behaviors or conditions that increase a disease.

2. History

The term "MetS" dates back to at least the late 1950s but came into common usage in the late 1970s to describe various risk factors associated with diabetes, something that had been noted as early as the 1920s (*6*,*7*).

i) In 1947, the Marseilles physician Dr. Jean Vague made the interesting observation that upper body obesity appeared to predispose one to diabetes,

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atherosclerosis, gout, and calculi (*8*).

ii) Avogaro, Crepaldi and their co-workers described six moderately obese patients with diabetes, hypercholesterolemia, and marked hypertriglyceridemia, all of which improved when the patients were put on a hypocaloric, low carbohydrate diet (*9*).

iii) In 1977, Haller used the term "MetS" for the association between obesity, diabetes mellitus, hyperlipoproteinemia, hyperuricemia, and steatosis hepatis when describing the additive effects of risk factors on atherosclerosis (*10*).

iv) The same year, Singer used the term for the association of obesity, gout, diabetes mellitus, and hypertension with hyperlipoproteinemia (*11*).

v) In 1977 and 1978, Gerald B. Phillips developed the concept that risk factors for myocardial infarction coincide to form a "constellation of abnormalities" (*i.e.*, glucose intolerance, hyperinsulinemia, hyperlipidemia [hypercholesterolemia and hypertriglyceridemia] and hypertension) that is associated not only with heart disease but also with aging, obesity and other clinical states. He suggested there must be an underlying linking factor, the identification of which could lead to the prevention of cardiovascular disease; he hypothesized that this factor was sex hormones (*12,13*).

vi) In his Banting lecture in 1988, Gerald M. Reaven proposed IR as an underlying factor and named the constellation of abnormalities Syndrome X. Reaven did not include abdominal obesity, which has also been hypothesized to be an underlying factor, as part of the condition (*14*).

3. Risk factors

The following factors increase chances of developing MetS.

3.1. *Age*

The risk of MetS increases with increasing age, affecting less than 10% of people in their 20s and 40% of people in their 60s. However, some research shows that about one in eight school children has three or more components of MetS. Other research has also identified an association between childhood MetS and adult cardiovascular disease decades later.

3.2. *Race*

About 47 million adults in the United States (almost 25%) have MetS. The condition is more common in African American women and Mexican American women than in men of the same racial groups. MetS affects Caucasian women and men roughly equally. Some racial and ethnic groups in the United States are at higher risk of MetS than others. Mexican Americans have the highest rate of MetS, followed by Caucasians and African Americans. In addition, certain ethnic groups, such as Hispanics and South Asians, are at increased risk for MetS.

3.3. *Obesity*

A body mass index (BMI), a measure of percentage of body fat based on height and weight, greater than 25 increases the risk of MetS. Excess fat in the abdominal area is a greater risk factor for heart disease than excess fat in other parts of the body, such as on the hips. Therefore, so does abdominal obesity, *i.e.*, having an apple shape rather than a pear shape.

3.4. *History of diabetes*

There is a greater likelihood of MetS if a family history of type 2 diabetes or a history of diabetes during pregnancy (gestational diabetes) is present.

3.5. *Other diseases*

A diagnosis of fatty liver, gallstones, breathing problems during sleep, cardiovascular disease, or polycystic ovary syndrome (such metabolic problems affect a woman's hormones and reproductive system) also increases the risk of MetS.

4. How is MetS diagnosed?

The risk factors seen in MetS include: IR, obesity (especially abdominal obesity), high blood pressure, high fasting glucose or hyperglycemia, and lipid abnormalities. There must be at least three of the following five metabolic risk factors for an individual to be diagnosed with MetS:

a) A higher TG level (≥ 150 mg/dL than normal). TGs are a kind of fat and hang out in fat cells but they also circulate in the blood.

b) A lower high density lipoprotein (HDL) cholesterol level (< 50 mg/dL for women and < 40 mg/dL for men than normal). HDL is sometimes called "good" cholesterol because it helps in removing cholesterol from arteries. It cleans out the low density lipoproteins (LDLs) or "bad" cholesterol from blood. When there are not enough HDLs, the LDLs can run rampantly, causing plaque to build up in artery walls and put a strain on the heart and circulatory system. A low HDL cholesterol level increases the risk of coronary heart disease.

c) Higher blood pressure (≥ 130/85 mmHg than normal). Blood pressure pushes the blood against the arterial walls as the heart pumps out blood. If this pressure rises and remains high, it can damage the heart and lead to plaque buildup.

d) Higher fasting blood glucose level (more than 100 mg/dL). Fasting blood glucose between 100 and 110 mg/dL is a sign of MetS. A mildly high blood sugar (between 100 and 125 mg/dL) may be an early predictor of diabetes. About 85% of people who have type 2 diabetes, the most common type of diabetes, also have MetS. These people have a much higher risk of heart disease than the 15% of people who have type 2 diabetes but not MetS.
e) Large waist circumference (≥ 35 inches for women and ≥ 40 inches for men). Having a large waist circumference or apple-shaped figure means that there is excess weight around the waist (abdominal obesity), representing an increased risk of heart disease and other health problems.

Throughout the years, several definitions of MetS have been proposed, emphasizing IR or abdominal/visceral obesity. However, the 4 main definitions are from the World Health Organization (WHO) definition 1999 (*15*), the Adult Treatment Panel III (ATPIII) Report 2001 (*16*), the European Group for the Study of IR (EGIR) 1999 (*17*), and the International Diabetes Federation (IDF) consensus (*18*) on MetS (Table 1). The definition of MetS according to the National Cholesterol Education Program (NCEP) was slightly updated by the American Heart Association (AHA) and National Heart, Lung, and Blood Institute (NHLBI) in 2005 (*19*,*20*) and the same year IDF proposed a new definition (*21*) based on clinical criteria. The two are very similar and should presumably identify many of the same individuals as having MetS. This modification of the IDF and ATP III definitions increased the emphasis on abdominal obesity as the core feature of MetS. The two differences are that the IDF definition excludes any subject lacking an increased waist circumference while the NCEP definition diagnoses MetS based on other criteria. Secondly, the IDF definition uses physical feature-specific cut-off points for waist circumference, while the NCEP definition uses only one set of cut-off points for waist circumference regardless of physical features. Abdominal obesity measured by waist circumference is an essential requirement for the diagnosis, while other variables featured in the ATP III definition have changed slightly (Table 1).

4.1. *WHO criteria* (*15*)

According to WHO criteria (1999), the presence of MetS requires the presence of diabetes mellitus, impaired glucose tolerance, impaired fasting glucose or IR, and at least two of the above factors.

4.2. *NCEP-ATP III criteria* (*16*)

The NCEP-ATP III definition (2001) requires at least three of the risk factors.

4.3. *EGIR criteria* (*17*)

The EGIR definition (1999) requires IR in the top 25% of the fasting insulin values among non-diabetic individuals and two or more risk factors.

4.4. *AHA/NHLBI/Updated NCEP criteria* (*19*,*20*)

There is confusion as to whether AHA/NHLBI intended to create another set of guidelines or simply update the NCEP-ATP III definition. According to Scott Grundy, University of Texas Southwestern Medical School, Dallas, Texas, the intent was just to update the NCEP-ATP III definition and not create a new definition.

5. Insulin resistance – A key aspect of MetS?

A key aspect of MetS is IR. In the body's attempt to counterbalance IR, extra insulin is produced, leading to increased insulin levels. The increased insulin levels can directly or indirectly lead to the characteristic metabolic abnormalities seen in patients. Frequently, the IR will progress to overt type 2 diabetes, further increasing the risk of cardiovascular complications.

6. Who is more susceptible to MetS?

MetS tends to run in families, along with the propensity for type 2 diabetes. MetS will occur in susceptible people who become overweight and sedentary. Therefore, MetS (like type 2 diabetes) can most often be prevented by exercise and maintaining a healthy body weight.

7. Current global status of MetS and cardiovascular disease

MetS is a cluster of metabolic risk factors that is strongly associated with the potential development of atherosclerotic cardiovascular disease (CVD) and type 2 diabetes mellitus (T2DM). The prevalence of MetS depends on age, ethnic background, and gender. It increases linearly from the age of 20 until age 50, when it plateaus. Global statistics show that approximately a quarter of the adult population suffers from this clinical condition. Various studies from around the world, including those of the general population, indicate that individuals age 20-25 and up have a prevalence of 24% (India), 28% (USA), 30.1% (Tehran), 33.4% (Turkey), and 39.3% (Saudi Arabia). A high prevalence of MetS has been reported in sub-Saharan Africa and the Middle East; South Africa, Morocco, Oman, and Iran have reported prevalence rates of 33.5%, 16.3%, 21%, and 33.7%, respectively. Prevalence rates are also high in Venezuela (31.2%) and urban Brazil (25.4%) (*22*,*23*). The situation appears to be similar in South Asian countries. The recent data show that one-

Table 1. Diagnostic criteria for metabolic syndrome

S. No.	Criteria for metabolic syndrome	Obesity		Dyslipidemia		Blood pressure (Systolic and Diastolic)	Glucose	Insulin resistance	Other
		Male	Female	Male	Female				
1.	WHO (5th or 6th + ≥ 2 criteria), 1999	WHR > 0.90 and/or BMI > 30 kg/m^2	WHR > 0.85 and/or BMI > 30 kg/m^2	TG ≥ 150 mg/dL (≥ 1.7 mM); HDL-C < 35 mg/dL (< 0.9 mM)	TG ≥ 150 mg/dL (≥ 1.7 mM); HDL-C < 39 mg/dL (<1.0 mM)	≥ 140/90 mmHg	T2DM, impaired glucose tolerance, impaired fasting glucose	IR measured under hyperinsulinemiceu glycemic conditions	Urinary albumin excretion rate ≥ 20 μg/min or albumin: creatinine ratio ≥ 30 mg/g
2.	EGIR (5th + ≥ 2 criteria), 1999	WC ≥ 94 cm	WC ≥ 80 cm	TG ≥ 177 mg/dL (≥ 2.0 mM); HDL-C < 39 mg/dL (< 1.0 mM)		≥ 140/90 mmHg or on medication	Fasting glucose > 110 mg/dL (≥ 6.1 mM)	IR	–
3.	NCEP-ATP III (≥ 3 criteria), 2001	Abdominal obesity WC ≥ 102 cm or 40 inches	Abdominal obesity WC > 88 cm or 36 inches	TG ≥ 150 mg/dL; HDL-C < 40 mg/dL or on therapy	TG ≥ 150 mg/dL; HDL-C < 50 mg/dL or on therapy	≥ 130/85 mmHg or on therapy	Fasting glucose > 110 mg/dL (≥ 6.1 mM)	–	–
4.	AHA/NHLBI or Updated NCEP criteria, 2005	WC ≥ 102 cm (Asian ≥ 90) or 40 inches	WC > 88 cm (Asian ≥ 80) or 36 inches	TG ≥ 150 mg/dL (≥ 1.7 mM); HDL-C < 40 mg/dL (1.0 mM) or on therapy	TG ≥ 150 mg/dL (≥ 1.7 mM); HDL-C < 50 mg/dL (1.0 mM) or on therapy	≥ 130/85 mmHg or on medication	Fasting glucose > 100 mg/dL (5.6 mM)	–	–
5.	IDF (1st + ≥ 2 other criteria), 2005	Ethnicity-specific WC (≥ 90 cm for men and ≥ 80 cm for women) for Indian subjects (If BMI is > 30 kg/m^2, central obesity can be assumed and WC does not need to be measured)		TG ≥ 150 mg/dL (≥ 1.7 mM); HDL-C < 40 mg/dL (1.03 mM) or on therapy	TG ≥ 150 mg/dL (≥ 1.7 mM); HDL-C < 50 mg/dL (1.3 mM) or on therapy	≥ 130/85 mmHg or on therapy	Fasting glucose > 100 mg/dL (5.6 mM) or DM	–	–

Abbreviation: Waist circumference (WC).

fourth to one-third of the urban population of India has MetS (*24*). MetS is highly prevalent among urban populations of Indians (35.2% *vs.* 20.6%) compared to rural populations. Its prevalence increases with age and is 1.5-2 times higher in women than in men (*24,25*). Interestingly, certain communities in India (*e.g.* the Punjabi Bhatia community in northern India) tend to have an inordinately high incidence of obesity, T2DM, and MetS (*26*). Two Indian studies described vastly different rates of MetS prevalence in India. Both had different definitions of obesity: one used obesity criteria tailored to Indians while the other (*27*) used the standard ATP III definition of obesity. Both studies used population-based samples within the same age range but reported prevalence of 13% in Jaipur (*27*) and 41% in Chennai (*28*). The studies had far larger differences in terms of the prevalence of elevated TG (46% *vs.* 30%), hypertension (55% *vs.* 39%), and elevated fasting plasma glucose (27% *vs.* 5%) although both reported having used the same cut-off points. Interestingly, a third Indian study (*29*), also of Chennai, reported a MetS prevalence of 11.2% (using EGIR criteria), which was much closer to the prevalence rate reported for Jaipur than that reported for Chennai. Therefore, there appear to be significant differences in the prevalence of both individual factors that constitute MetS and MetS itself even within the same ethnic population. Hispanics and African-Americans have the greatest risk for developing MetS, followed by Caucasians. Asians have the lowest risk, at least in the United States. The prevalence of MetS, when based on the ATP III criteria, varies as described earlier among ethnic groups like Finnish and Native American men. Studies of these two groups involved subjects with comparable age ranges (42-60 and 44-49 years, respectively), and yet the Finnish study found a prevalence of only 14% compared with a prevalence of 43.6% in the study of Native Americans. Prevalence rates vary from a low of 13.9% in black men to a high of 27.2% in Mexican American women (*30*). The literature indicates that MetS is currently more prevalent and a danger to a large number of people worldwide.

CVD causes numerous deaths worldwide and in India. People with MetS are at higher risk of morbidity and mortality from CVD (*31*). The main reason for this is that the combination of MetS risk factors interacts synergistically to start or accelerate the progression of atherosclerosis (*31*). At the same time, early diagnosis, prevention, and management of MetS are considered the key approaches in reducing the risk of progression of atherosclerosis and development of CVD (*32*). One report suggested that MetS could be responsible for approx 7% of total mortality, regardless of the cause, and up to 17% of CVD (*33*). Similarly, a report from the Framingham Heart Offspring Study showed that the contribution of MetS to the risk of CVD was 34% in men and 16% in women (*34*). In that analysis, the components of the syndrome that contributed most to the CVD outcomes were high blood pressure (33%) and low HDL-cholesterol (25%). A meta-analysis of 37 longitudinal studies found a 78% increased risk for CVD events and death in people with MetS (*35*). The ability of CVD to predict MetS may vary by ethnicity, gender, and the presence or absence of hyperglycemia. Moreover, few studies have investigated the mortality rate, lifestyle behaviors, and nutritional factors that are known to be risk factors for MetS and CVD in India (*36*). Major differences in CVD mortality rates in different Indian states were reported, varying from 75-100 per 100,000 people in sub-Himalayan states of Nagaland, Meghalaya, Himachal Pradesh, and Sikkim to a high of 360-430 in Andhra Pradesh, Tamil Nadu, Punjab, and Goa. The pressing issues with regard to MetS and CVD in India is that the prevalence of the two conditions has increased dramatically and that there is no clear information on the risk factors for MetS and CVD among the population of Northern India. MetS therefore may become a major contributor to accelerated aging and functional decline and could represent a major public health problem, eclipsing CVD and type 2 diabetes in the near future.

8. Risk of developing CVD in individuals with MetS

The relative risk of developing CVD associated with MetS as defined by NCEP-ATP III or by other organizations has increased 2- to 5-fold in both men and women and in various populations (*31,37,38*). Data from a Quebec cardiovascular study of individuals with several risk factors associated with MS were characterized by a tremendous increase in the relative risk of CVD compared to individuals who had only one or none of the risk factors. For example, non-diabetic men who had hyperapoB, small dense LDL, and hyperinsulinemia simultaneously had a 20-fold increase in the risk of CVD over 5 years compared to men who had none of these metabolic perturbations (*39*).

Another study showed that the risk associated with hypertriglyceridemia was modulated to a significant extent by the presence or absence of other components of MetS. For example, men with marginally increased plasma TG levels (above 1.6 mM) but with no other features of IRS had a 3-fold increase in the risk of CVD compared to men with normal plasma TG levels (*40*). The risk of CVD increased 13-fold for subjects with moderate hypertriglyceridemia who also had hyperapoB, reduced HDL-C levels, and increased insulin concentrations (*40*). These data clearly indicate that MetS, irrespective of its definition, may be associated with a significantly increased risk of CVD. Therefore, components of MetS may significantly contribute to this increased risk of CVD.

9. Etiology

The cause of MetS is unknown. Its pathophysiology is extremely complex and has been only partially elucidated. Most patients are older, obese, sedentary, and have a degree of IR. The most important factors are, in order: *i*) aging, *ii*) genetic makeup, and *iii*) daily lifestyle and habits (*e.g.* low physical activity and excess caloric intake).

There is debate regarding whether obesity or IR is the cause of MetS or if obesity and IR are consequences of more far-reaching metabolic dysfunction. A number of markers of systemic inflammation, including C-reactive protein (CRP), often increase, as do interleukin 6 (IL-6), tumor necrosis factor-alpha (TNF-α), resistin, leptin, and adiponectin.

10. Pathophysiology

Obesity and metabolic abnormalities were known to be associated with poor cerebrovascular outcomes when the concept of MetS first appeared. In 1995, Dr. G Reaven noticed that those outcomes were found in people who had hyperinsulinemia, high TG, low HDL cholesterol, and hypertension, all of which were considered factors for the development of CVD. Many earlier studies measured only serum total cholesterol regardless of LDL-cholesterol levels, although most total cholesterol consists of LDLs. Thus, the robust relationship between total cholesterol and coronary heart disease found in epidemiological studies strongly implies that an elevated LDL is a highly prevalent and powerful risk factor. Epidemiological investigations of human populations point to high levels of LDL cholesterol as being atherogenic lipoprotein.

Previous research demonstrated that adipose tissue plays an important role in energy regulation *via* endocrine, paracrine, and autocrine signals (*41*) and various factors known as 'adipokines' released by adipose cells cause IR. These adipokines are defined as insulin antagonists (TNF-α, IL-6, and resistin) and insulin sensitizers (leptin and adiponectin) (*42*). These adipokines markedly affect peripheral functions and influence the pathogenesis of obesity-related disease, particularly diabetes and cardiovascular disorders.

Visceral fat accumulation is found to be specifically associated with metabolic alteration of obesity in both men and women. Increasing accumulation of visceral fat leads to the overproduction of some adipokines such as IL-6, TNF-α and resistin, decreasing insulin action in muscles and/or the liver, while some adipokines like leptin and adiponectin have a beneficial effect on energy balance, insulin action, and vasculature. Leptin regulates energy balance and has an insulin-sensitizing effect. These beneficial effects are reduced in obesity due to leptin resistance. Adiponectin increases insulin action in muscles and the liver and has an anti-atherogenic effect. Conversely, excessive production of other adipokines is deleterious. The increased levels of circulating adipokines associated with visceral obesity may be attributed to production by ectopic adipose tissue. Adiponectin is the only known adipokine with circulating levels that decrease as a result of visceral obesity while levels of other adipokines increase. This dysregulation of adipokine production may promote obesity-linked metabolic disorders and CVD.

The accumulation of visceral fat hastens the release of non-esterified fatty acids (NEFAs), resulting in greater lipolytic activity in obese individuals and increasing NEFA levels in systemic circulation. This increased release of NEFAs into the portal circulation stimulates hepatic glucose production and reduces hepatic insulin clearance, ultimately resulting in insulin resistance, hyperinsulinemia, and hyperglycemia (*43*). When obesity develops, abnormal production of these adipokines by more visceral fat contributes to a proinflammatory state. This state of inflammation is likely to contribute to the health problems associated with obesity such as dyslipidemia, insulin resistance, and atherosclerosis. In contrast to these adipokines, levels of the insulin sensitizing and anti-inflammatory adipokine adiponectin are reduced in visceral obesity (*44*), and this may further exacerbate the state of low-grade systemic inflammation. In the liver, adiponectin increases insulin sensitivity by lowering NEFA uptake, increasing fatty acid oxidation, and reducing hepatic gluconeogenesis and very low density lipoprotein (VLDL) production. In muscle, adiponectin stimulates glucose uptake and fatty acid oxidation (*45*). Therefore, the altered expression of adipokines associated with visceral obesity induces a state of low-level systemic inflammation and dyslipidemia, eventually leading to atherosclerosis. In visceral obesity, dysregulated production of specific adipokines may contribute to hypertension and CVD. Hypertension is a common manifestation of the metabolic disturbances associated with insulin resistance and hyperinsulinemia, a key factor for MetS. Studies using the hyperinsulinemic-euglycemic clamp technique have demonstrated that hyperinsulinemia occurs in hypertension as a compensatory response to reduced insulin-stimulated glucose uptake by skeletal muscle (*46*,*47*). Adipocytes synthesize and release several factors that have been linked to blood pressure control, including adiponectin, leptin, and resistin. Increasing evidence suggests that aberrant production and release of such adipokines as adiponectin, leptin, and resistin by adipocytes may contribute to the high prevalence of hypertension in visceral obesity. Therefore, adipokines are potential causes of insulin resistance, endothelial dysfunction, and hypertension and reflect the role visceral obesity plays as a causal factor in metabolic and vascular disease.

11. Treatments and drugs for MetS

Tackling one of the risk factors for MetS is tough – taking them all on might seem overwhelming. That says, healthy or aggressive lifestyle changes and, in some cases, medication can improve every component of MetS. Lifestyle changes include losing weight, getting more regular physical activity, following a heart healthy diet, quitting smoking, reducing one's blood pressure, and improving one's cholesterol and blood sugar levels. The main focus of treating MetS is managing the risk factors that are under control, such as being overweight or obese, having an inactive lifestyle, or consuming an unhealthy diet. These changes are the key factors in reducing metabolic risk.

11.1. *Exercise*

More activity means more of a benefit. The four main types of physical activity are aerobic, muscle strengthening, bone strengthening, and stretching. Physical activity can be light, moderate, or vigorous. Doctors recommend 30 to 60 min of moderate-intensity exercise, such as brisk walking, every day.

11.2. *Losing weight*

In general, people who have MetS and are overweight or obese and who then lose as little as 7-10% of their body weight can reduce their insulin levels and blood pressure and decrease their risk of diabetes.

11.3. *Lipid abnormalities*

While the lipid abnormalities seen with MetS (low HDL, high LDL, and high TGs) respond nicely to weight loss and exercise, drug therapy is often required. Treatment should be aimed primarily at reducing LDL levels according to specific recommendations. Once reduced LDL targets are reached, efforts should be made to reduce TG levels and raise HDL levels.

11.4. *Clotting disorders*

People with MetS can have several coagulation disorders that facilitate the forming of blood clots within blood vessels. These blood clots are often a precipitating factor for a heart attack. Excessive blood clotting is a condition that often occurs with MetS.

11.5. *Eating healthy*

A heart healthy diet is an important part of a healthy lifestyle. The Dietary Approaches to Stop Hypertension (DASH) diet and the Mediterranean Diet, like many healthy-eating plans, limit unhealthy fats and emphasize fruits, vegetables, fish, and whole grains. Both of these dietary approaches have been found to offer important health benefits – in addition to weight loss – for people who have components of MetS. A doctor's guidance is needed before starting a new eating plan.

11.6. *Stopping smoking*

Smoking can increase the risk of heart disease and heart attacks and worsen other heart disease risk factors. Smoking cigarettes increases IR and worsens the health consequences of MetS. A doctor should be consulted for help in quitting cigarettes. The doctor can help the individual to monitor weight and blood glucose, cholesterol, and blood pressure levels in order to ensure that lifestyle modifications are working.

11.7. *Medication*

If goals cannot be achieved with lifestyle changes, a doctor may also prescribe medications to lower blood pressure with diuretics or angiotensin-converting enzyme (ACE) inhibitors, reduce unhealthy cholesterol levels with statins, fibrates, or nicotinic acid, or provide help in losing weight. High blood sugar is treated with oral medicines such as metformin, insulin injections, or both. Low-dose aspirin can help reduce the risk of blood clots, especially for people at high risk of heart disease.

12. Conclusion

Both an increased waist:hip ratio and IR are emerging risk factors for MetS, and Indians are considered to be more genetically predisposed to both. Larger numbers of people with MetS are linked to a rise in obesity rates among adults. In the future, MetS may overtake smoking as the leading risk factor for heart disease. The key to preventing MetS, however, remains diet and exercise. Any person with a strong family history of MetS or type 2 diabetes should be especially careful to maintain a healthy lifestyle. A healthy lifestyle is a lifelong commitment. Successfully controlling MetS takes a long-term effort and teamwork with health care providers.

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Brief Report

The treatment effect of the burn wound healing by electrolytic-reduction ion water lotion combination therapy. Part 2: Two degree burn of forearm to the dorsum of the hand

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Summary **Patient 1: A 1-year-and-3-month-old boy suffered a burn injury extending from the left forearm to hand due to boiling water. An extensive skin defect from the left forearm to the dorsum of the hand was observed, and an IIb-III degree burn was diagnosed. Treatment of the burn was started with the application of electrolytic-reduction ion water (ERI) lotion, antibiotic/steroid combination ointment, and vitamin A/E ointment with wrap therapy. Two days after the initiation of therapy, redness and swelling were still observed despite a slight decrease in swelling. After 21 days, skin redness decreased, and there was no functional impairment. After 74 days, the skin color was almost normal, and no functional impairment was observed, showing a favorable course.**

Patient 2: An 8.5-month-old girl suffered a burn injury extending from the fingers to dorsum of the right hand and right wrist due to boiling water. There was an extensive skin defect accompanied by bulla formation extending from the fingers and dorsum of the right hand. An II-degree burn was diagnosed. Treatment of the burn was started treatment similar to the Patient 1. Bulla decreased, but redness and swelling were still present 2-8 days after the initiation of therapy. After 16-25 days, both skin redness and swelling decreased. After 30 days, the epithelialization of the dorsum of the hand had almost completed. After 60 days, the skin color was nearly normal, and there was no functional impairment, showing a favorable course.

In these patients, burn wounds completely healed without hypertrophic or keloid scar formation or pigmentation. These results suggest that extensive II-III burns can be adequately treated by this topical therapy.

Keywords: Electrolytic-reduction ion water (ERI), burn wound, moist wound healing, conservative treatment, wrap therapy

1. Introduction

Major surgical primary emergency disorders in children include injuries, burns, inflammatory diseases, and the accidental ingestion of foreign bodies (*1,2*). Among them, the number of burn cases is high, and burns in infants are often severe. In the treatment of II and III burns, local therapy with disinfectants and ointments containing steroids or antibiotics was conventionally performed to inhibit infection of the wound surface. However, in recent years, this conventional therapy has been gradually changed to occlusive dressing therapy with wound dressings (such as polyurethane films, hydrocolloid, polyether foam, and hydro-gel) that maintains the moist environment in the burn area and optimizes the repair function of the body (*3-5*). In addition, studies have shown that plastic films for food wrapping (wrap films) can be inexpensive materials appropriate for dressing, and therapy using wrap films (wrap therapy) is an effective burn treatment method (*6,7*).

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Electrolytic-reduction ion water (ERI) is water physically abundant in extra electrons produced by the electrolysis of natural water. Due to its special alkaline property and negative ions, ERI shows various favorable effects such as cleaning, disinfectant, antioxidant, and emulsifying effects (*8-11*).

In this study, in patients 1 and 2, wrap therapy as conservative treatment with an ERI lotion, antibiotic/steroid combination ointment, and vitamin A/E ointment was performed, and favorable results could be obtained.

2. Materials and Methods

2.1. *Materials*

ERI lotion manufactured by A.I. System Products Co., Aichi, Japan (S-100, 94.9%; glycerin, 3%; ascorbic acid, 2%; hyaluronic acid, 0.1%) was used. As antibiotic/steroid combination ointments, Hysetin-P® ointment (containing chloramphenicol, fradiomycin sulfate, and prednisolone) and Dexan-G® ointment (containing betamethasone valerate and gentamicin sulfate) manufactured by Fuji Pharma Co., Ltd. (Shizuoka, Japan) were used. As a vitamin A/E ointment, Juvela® ointment (containing tocopherol and vitamin A oil) manufactured by Eisai Co., Ltd. (Tokyo, Japan) was used.

2.2. *Methods*

The burn was treated by the application of ERI lotion, antibiotics/steroid combination ointment (Hysetin-P or Dexan-G), and vitamin A/E ointment (Juvela) to the burn area 3 times/day combined with wrap therapy, *i.e.*, wrapping the injury site with a thin plastic film to prevent drying (*12*).

3. Results and Discussion

3.1. *Patient 1*

A male aged 1 year and 3 months sustained burn injury to the dorsum of the hand and was treated on an outpatient basis in another hospital. Four days after injury, he was taken to our hospital. There was an extensive skin defect over the entire dorsum of the hand. A diagnosis of a IIb-III burn was made. The treatment course from the first consultation day to 130 days after the initiation of treatment is shown in Figure 1.

Figure 1A shows the state on the first consultation day. Marked redness and swelling of the fingers and dorsum of the left hand and the left wrist were observed. The central area of the dorsum of the hand was whitish. The skin was detached from the lower 1/2 area of the fingers to the entire dorsum of the hand. Therefore, an ERI lotion, antibiotic/steroid combination ointment (Hysetin-P ointment), and vitamin A/E ointment (Juvela ointment) were applied to the burn area, and wrap therapy was initiated. Figure 1B shows the state 2 days after the initiation of therapy. Redness and swelling were still observed despite a slight reduction in swelling. After 3-6 days, redness and swelling were still present (Figures 1C-1E). After 7 days, Hysetin-P ointment as an antibiotic/steroid ointment was changed to Dexan-G ointment, and burn treatment was continued employing a similar method (Figure 1F). Figure 1G shows the state after 10 days. Redness and swelling persisted despite a slight reduction in redness. Figure 1H shows the state after 16 days. Skin redness further reduced, and swelling decreased. Figures 1I and 1J show the states after 17 and 21 days, respectively. Skin redness further decreased, and there was no functional impairment. After 32 days, the epithelialization of the dorsum of the hand was completed (Figure 1K). Figures 1L and 1M show the states after 44 and 74 days, respectively. Despite slight skin redness, no functional impairment was observed, showing a favorable course. Redness was negligible 98 days after the initiation of therapy (Figure 1N). After 130 days, there was neither scar formation nor pigmentation, and the skin color was normal in appearance. Therefore, the burn wound was considered to have completely healed (Figure 1O).

3.2. *Patient 2*

The treatment course from the first consultation day to 111 days after the initiation of treatment is shown in Figure 2. On the first consultation day, marked redness and swelling accompanied by bullae were observed in the fingers-dorsum of the right hand and right wrist. In particular, the degree of the burn was IIa-IIb in the right thumb, index finger, middle finger, and the central area of the dorsum of the hand (Figure 2A). Therefore, an ERI lotion, antibiotic/steroid combination ointment (Hysetin-P ointment), and vitamin A/E ointment (Juvela ointment) were applied to the burn area, and wrap therapy was initiated. After 2-8 days, bullae decreased, but redness and swelling were still present (Figures 2B-2G). Therefore, Hysetin-P ointment as an antibiotic/steroid combination ointment was changed to Dexan-G ointment, and burn treatment was continued employing a similar method (Figure 2H). After 13 days, redness and swelling were present despite a slight decrease in redness (Figure 2I). After 16-25 days, both skin redness and swelling decreased (Figures 2J-2L). After 30 days, the epithelialization of the dorsum of the hand was nearly completed (Figure 2M). After 60 days, the skin color was almost normal, and no functional impairment was observed, showing a favorable course (Figure 2N). After 111 days, there was a slight scar but no pigmentation, and the skin color was normal in appearance. Therefore, the burn

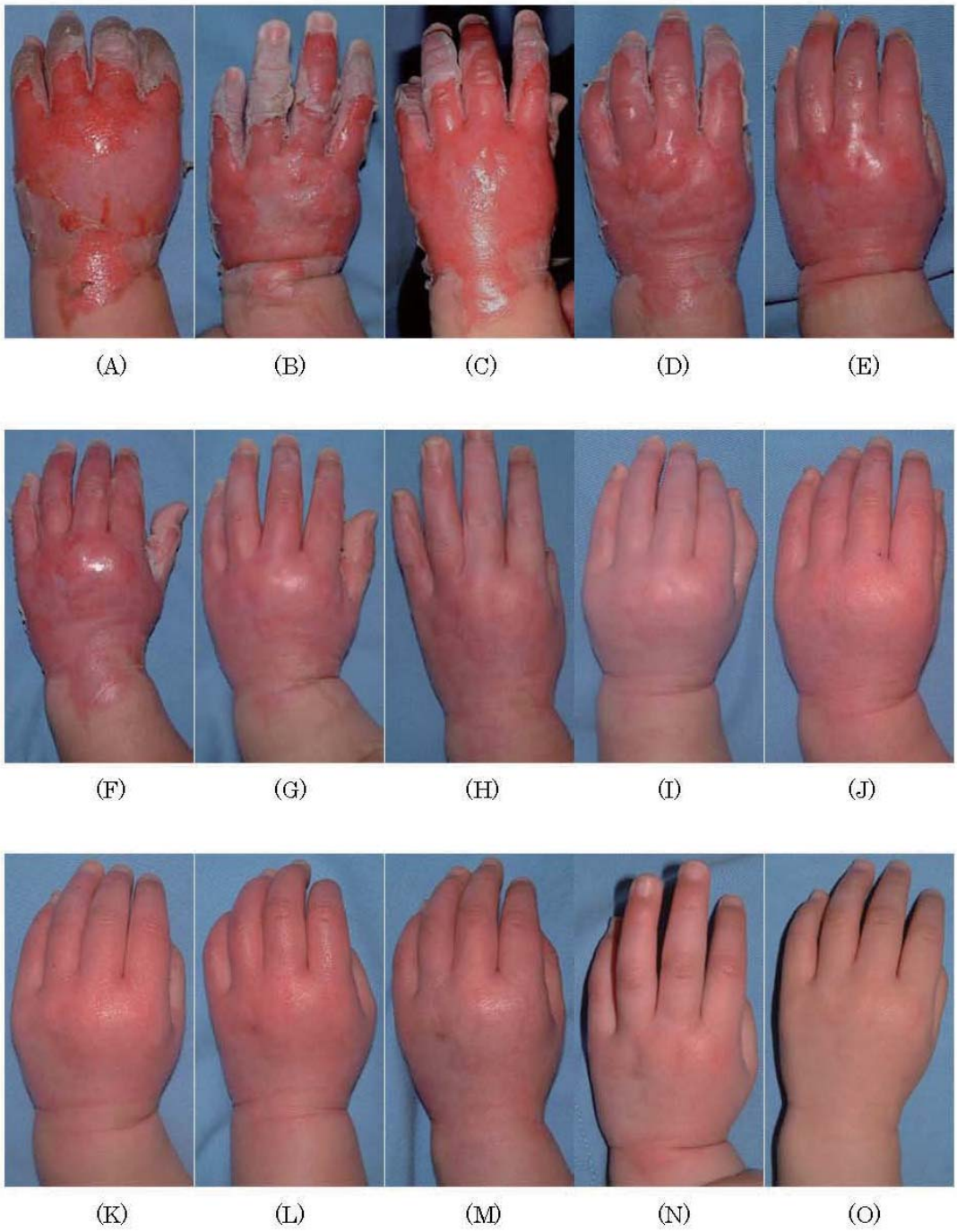

Figure 1. Patient 1: A case involving treatment of the dorsum of the left hand with ERI lotion, antibiotic/steroid combination ointment, and vitamin A/E ointment employing wrap therapy. (A) Before treatment. **(B)** 2nd day after treatment. **(C)** 3rd day after treatment. **(D)** 5th day after treatment. **(E)** 6th day after treatment. **(F)** 7th day after treatment. **(G)** 10th day after treatment. **(H)** 16th day after treatment. **(I)** 17th day after treatment. **(J)** 21st day after treatment. **(K)** 32nd day after treatment. **(L)** 44th day after treatment. **(M)** 74th day after treatment. **(N)** 98th day after treatment. **(O)** 130th day after treatment.

wound was considered to have completely healed (Figure 2O).

Burn injuries often require surgical skin grafting depending on their depth in addition to conventional topical therapy. Our patients had II-III burns. However, since their parents hoped for treatment on an outpatient basis, wrap therapy as conservative treatment with an ERI lotion, antibiotic/steroid combination ointment, and vitamin A/E ointment was performed. In these patients, Hysetin-P ointment was changed to Dexan-G ointment 7-9 days after the initiation of therapy. This was because ointments showing the more marked anti-inflammatory activity of topical steroids were considered to be more effective.

In both patients, the tissue repair course for burn healing smoothly advanced, resulting in complete healing without hypertrophic or keloid scar formation

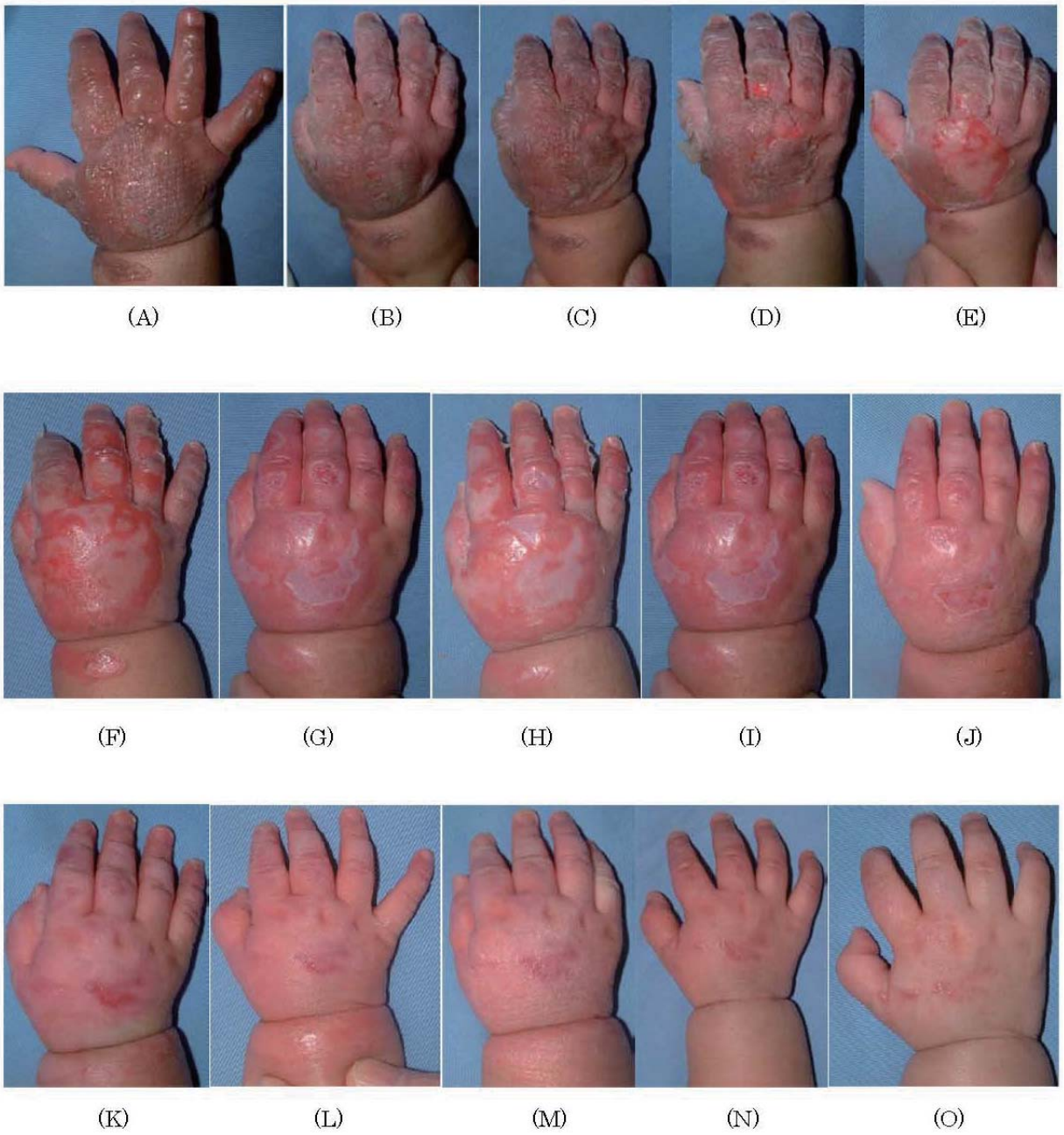

Figure 2. Patient 2: A case involving the treatment of all fingers and the dorsum of the right hand with ERI lotion, antibiotic/steroid combination ointment, and vitamin A/E ointment employing wrap therapy. (A) Before treatment. **(B)** 2nd day after treatment. **(C)** 3rd day after treatment. **(D)** 4th day after treatment. **(E)** 5th day after treatment. **(F)** 6th day after treatment. **(G)** 8th day after treatment. **(H)** 9th day after treatment. **(I)** 13th day after treatment. **(J)** 16th day after treatment. **(K)** 20th day after treatment. **(L)** 25th day after treatment. **(M)** 30th day after treatment. **(N)** 60th day after treatment. **(O)** 111th day after treatment.

or pigmentation. These results suggest that extensive II-III burns can be adequately treated by topical therapy with an ERI lotion, antibiotic/steroid combination ointment, and vitamin A/E ointment.

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Original Article

Health resource allocation and productive efficiency of Chinese county hospitals: Data from 1993 to 2005

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Summary **This study aims to assess trends in the productive efficiency of China's county hospitals during the economic transition using data from 1993 to 2005. A data envelopment analysis (DEA) framework was used to calculate the efficiency score of county hospitals in all 31 provinces. A C^2R model and a BC^2 model were devised to respectively calculate overall and scale efficiency and pure technical efficiency at the hospital's current scale. Models included four inputs (number of medical staff; number of beds; value of fixed capital; and hospital expenditures) and three outputs (outpatient and emergency visits, number of inpatients, and hospital revenue) in total. As the results, geographical disparities in health resource allocation and county hospital productivity were noted. From 1993 to 2005, the number of county hospitals increased and their inputs, *e.g.* fixed capital in particular, grew rapidly. However, the amount of both outpatient and inpatient services declined somewhat especially in the middle and the western regions. The overall efficiency at the national level decreased slightly. County hospitals in the eastern region tended to have better overall, scale, and technical efficiency in comparison to the middle and the western regions. In conclusion, county hospitals are inefficient due to their enlarged scale and the reduced amount of health care services they provide. This issue should be addressed especially in the middle and the western regions, where health resources are far more limited and yet wasted. The effects of ongoing health sector reform on the productivity of county hospitals must be monitored and evaluated.**

Keywords: County hospital, productivity, data envelopment analysis (DEA), China

1. Introduction

Productive efficiency is a key determinant of health system performance. It is about producing the maximum amount of health services, and ultimately health outcomes, from an available quantity of health system inputs, including finances, infrastructure, and human resources. As hospitals are the main providers of health care services and account for a large share of health expenditures, their productive efficiency must be monitored and evaluated as part of the policymaking process in order to optimize the utilization of available health resources and mobilize additional resources for the health system through increased efficiency.

In China, the economic transition from a central planned economy to a market-driven economy has resulted in unprecedented economic growth over the past three decades. That said, the equitable distribution of the benefits of this economic growth remains a concern, as disparities in income and wealth between urban and rural populations and between the east and the west have substantially widened. The economic transition also brought about profound changes in the health system, such as a decreased reliance on state

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funding, decentralization of public health services, increased hospital autonomy, and freedom of movement of health workers (*1-3*). Market-oriented health sector reform coincided with a soaring burden of individual medical expenses and a huge health gap paralleling socioeconomic and geographical status (*4-8*). According to World Health Report 2000, China's health system ranked 144th, and among the five performance indicators its fairness of financial distribution in the health system ranked 188th among the 191 countries in the World Health Organization (WHO) (*9*).

The Chinese government is now highly aware of these issues and has actively responded to them. A new reform seeking reasonable distribution of health resources and emphasizing core issues of equity and accessibility has been launched by infusing a large amount of public finances. This action plan emphasizes services at the rural and grass roots level, such as infrastructure and human resource development of the three-tiered network at the county, town, and village levels (*10*).

County hospitals are the main provider of a large share of services including treatment and emergency services, disease prevention, vaccination, health education, maternal and child health care services, and reproductive services for rural residents (*11*), who account for 80% of the Chinese population overall. These hospitals serve as the flagship in the three-tiered rural health care system. They take in 25.44% of basic and 36.19% of special government subsidies for the rural health care system (*12*). Like other hospitals, county hospitals were given a great deal of autonomy with no public finances as part of the market-oriented reform of the health sector. The productive efficiency of county hospitals must be comprehensively investigated in terms of geographical disparities, trends in health resource allocation and health services provision, and possible increased efficiency in order to rationally allocate health resources to the rural health system and effectively provide health care services to rural residents. Previous studies on the efficiency of Chinese hospitals were mainly small-scale investigations (*13,14*) while studies on efficiency over time at the national level are lacking. This study sought to assess the productive efficiency of Chinese county hospitals, to examine trends in health resource allocation and health services provision, and to explore the potential for increased efficiency in order to provide policymakers with evidence of improved performance.

2. Methods

2.1. *Data sources and hospitals studied*

Data are from the Health Service Management Budget Accounting Report in 1993, 1995, 1997, and 1999 and the Annual National Health Financing Report in 2001, 2003, and 2005. Both reports are issued by the Ministry of Health and have similar content.

The hospitals studied were all county hospitals in all 31 provinces, autonomies, and municipalities of mainland China. Based on their geographical location and socioeconomic status indicated by GDP per capita and average income, those provinces are officially divided into three groups in general: the developed eastern region, the moderately developed middle region, and the least developed western region.

2.2. *Efficiency and DEA framework*

As a non-parametric method first developed by Charnes *et al.* (*15*), data envelopment analysis (DEA) incorporates multiple output and input variables to score the efficiency of a decision-making unit (DMU), or county hospitals in each province, relative to a group of DMUs. It constructs a production possibilities frontier through the best performance of DMUs at different scales of operation. County hospitals comprising the best practice frontier are assigned an efficiency score of 100%. The efficiency of those below the frontier is measured by their distance from it. Inefficient county hospitals are assigned a score between one and zero. For hospitals with inefficiency, DEA can also indicate the amounts of over-supplied inputs or under-produced outputs in order to achieve maximum productive efficiency.

In this study, a C^2R model was used to calculate overall efficiency and a BC^2 model was used to calculate pure technical efficiency at the hospital's current scale, as described below:

$$Max\ h_0 = \frac{\sum_{r=1}^{s} u^T y_{rj0}}{\sum_{i=1}^{m} u^T x_{rj0}}$$

Subject to

$$\frac{\sum_{r=1}^{s} u^T y_{rj0}}{\sum_{i=1}^{m} u^T x_{ij0}} \le 1, \quad j = 1, 2, ..., n$$

$$u \ge 0, r = 1, 2, ..., s \text{ and } v \ge 0, i = 1, 2, ..., m \qquad \text{Eq.1}$$

Where y_{rj} = the amount of output r produced by county hospital j, x_{ij} = the amount of input m used by county hospital j, s = the sort of output, m = the sort of input, u_r = the weight given to output r, v_i = the weight given to input i, n = the number of county hospitals, j_0 = county hospitals studied.

Model 1 is a fractional programming model that can be converted into the linear forms of C^2R model (model 2) and BC^2 model (model 3) so that linear programming can be applied.

$$min\ [\theta - \xi\,(e'^T s^- + e^T s^+)],$$

$$\sum_{j=1}^{n} x_j \lambda_j + s^- = \theta x_0, \quad \sum_{j=1}^{n} y_j \lambda_j + s^+ = y_0,$$

$$\lambda_j \ge 0, j = 1, 2, ..., n, s^- \ge 0, s^+ \ge 0, \theta \in E \qquad \text{Eq.2}$$

Where θ = the factor by which an input set x_0 is adjusted to attain the minimum input level x_j in county hospital j, in order to reach the efficient frontier, ξ = infinitesimal variable, λ = intensity variables to identify efficient production, s^- = the vector variable of inputs, s^+ = the vector variable of outputs,

Based on model 2, overall efficiency is such that if $\theta = 1$, DMU-j is efficient; if $\theta < 1$, DMU-j is inefficient. Regarding scale efficiency,

$$K = \sum_{j=1}^{n} \lambda_j,$$

where K is the value of the return to scale for DMU-j. An increasing return to scale exists if K is less than one (K < 1), a constant return to scale if K is equal to one (K = 1), and a decreasing return to scale if K is greater than one (K > 1).

$$min\ [\theta - \xi\,(e^{'T}s^- + e^T s^+)],$$
$$s.t. \sum_{j=1}^{n} x_j\lambda_j + s^- = \theta x_0,\ \sum_{j=1}^{n} y_j\lambda_j + s^+ = y_0$$
$$\sum_{j=1}^{n} \lambda_j = 1$$
$$\lambda_j \geq 0, j = 1, 2, ..., n, s^- \geq 0, s^+ \geq 0, \theta \in E \qquad \text{Eq.3}$$

The production technology set of this BC^2 model is described as:

$$T_{BC^2} = \{(x,y) | x \geq \sum_{j=1}^{n} x_j\lambda_j,\ y \leq \sum_{j=1}^{n} y_j\lambda_j,\ \lambda_j \geq 0, j = 1, 2, ..., n\}$$

In model 3, pure technical productivity of DMU-j is efficient when $\theta = 1$ while it is inefficient when $\theta < 1$.

2.3. *Input and output indicators*

The DEA model was estimated with seven variables in total: four inputs and three outputs. The four inputs were labor, constructed in terms of the number of medical staff; the number of beds; value of fixed capital; and hospital expenditures. The three outputs included outpatient and emergency visits, number of inpatients, and hospital revenue. The selection of these input and output indicators was based on availability of data, a conceptual framework of hospital management, and consultation with an expert panel.

2.4. *Unit cost calculation*

Unit costs of outpatient and emergency services and inpatient services are calculated as below: Costs of inpatient services = (Fixed Capital × 0.125 + Medical expenditures) × 40%/outpatient and emergency visits; Costs of outpatient services = (Fixed Capital × 0.125 + Medical expenditures) × 60%/days beds were occupied.

With a depreciation rate of 0.125, outpatient and emergency services and inpatient services were estimated, based on Financial Regulations for Hospitals (*16*), to account for 40% and 60% of hospital expenditures, respectively.

2.5. *Discounting*

To facilitate comparison of different years, data on currency values such as the value of fixed capital, medical income, and expenditures were discounted based on the retail sale index (Table 1) (*17*). The baseline was the year of 2005.

3. Results

In general, the number of county hospitals increased from 2,696 hospitals in 1993 to 3,391 hospitals in 2005. The average annual increase in the eastern region, the middle region and the western region was 2.19%, 2.42% and 1.26%, respectively, and the number increased 1.93% for the country overall.

3.1. *Trends in health resource allocation*

The average number of medical staff in county hospitals has increased slightly. The average annual increase in the eastern, middle, and western regions was 1.97%, 0.56% and 0.44%, respectively, and 1.14% for the country overall.

Fixed capital included buildings and equipment. Its overall value rose rapidly during the 12 years studied, as the average annual increase reached 15.9%

Table 1. Retail sale index from 1993 to 2005

Year	Retail sale index
1993	70.94
1995	99.11
1997	105.98
1999	100.14
2001	97.86
2003	96.49
2005	100.00

Source: National Statistics 2006.

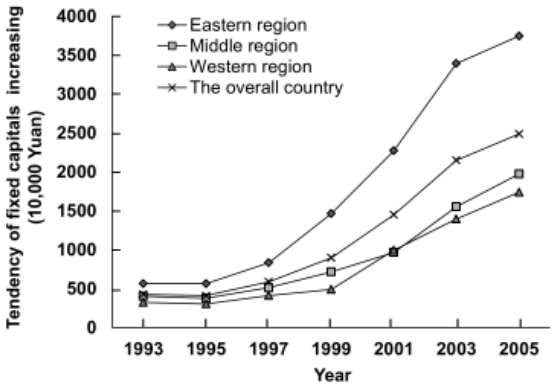

Figure 1. Rapid increase in fixed capital.

and the value in 2005 was 5.84 times that in 1993 (Figure 1). The average annual increase of the value of buildings was 12.0% for the country overall. The value of equipment grew even faster, as the average annual increase remained almost 20% and the value in 2005 was 8.8 times that in 1993.

At both the national level and provincial level, the number of beds gradually increased. The percentage of beds occupied decreased from 71.8% in 1993 to 59.6% in 2005 (Figure 2), and in almost all provinces this percentage was far below 84%, the standard set by the Ministry of Health.

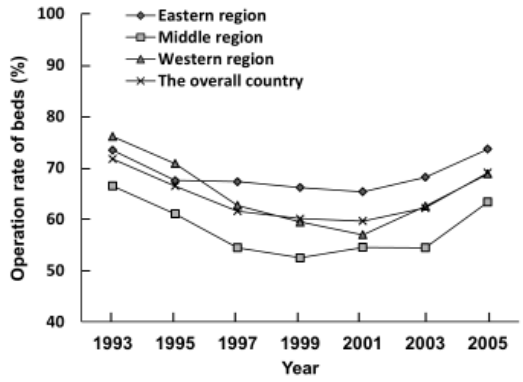

Figure 2. Beds operancy.

3.2. *Amounts and costs of medical services*

The number of average annual outpatient and emergency visits for the country overall decreased from 94,000 in 1993 to 74,000 in 1999 and then rose to 90,000 in 2005. The average number of inpatients also tended to decline annually particularly in the middle and western regions. The cost of both outpatient and emergency services and inpatient services increased 5 times from 1993 to 2005, with an average rate of 14.5% and 14.4% at the national level, respectively. The western region had the most rapid increase in outpatient and emergency services, 16.3% (Figure 3).

From 1993 to 2005, county hospitals in China had double-digit increases in revenue and expenditures of 13.5% and 13.6%, respectively. Most county hospitals, and especially those in the middle and the western regions, had a severe deficit (Figure 4). At the national level, government subsidies accounted for 7% of county hospital revenue.

3.3. *Productive efficiency based on DEA models*

3.3.1. *Overall efficiency*

Generally, overall efficiency at the national level decreased slightly from 1993 to 2005 (Figure 5). In 2005, there were 16 provinces where the overall

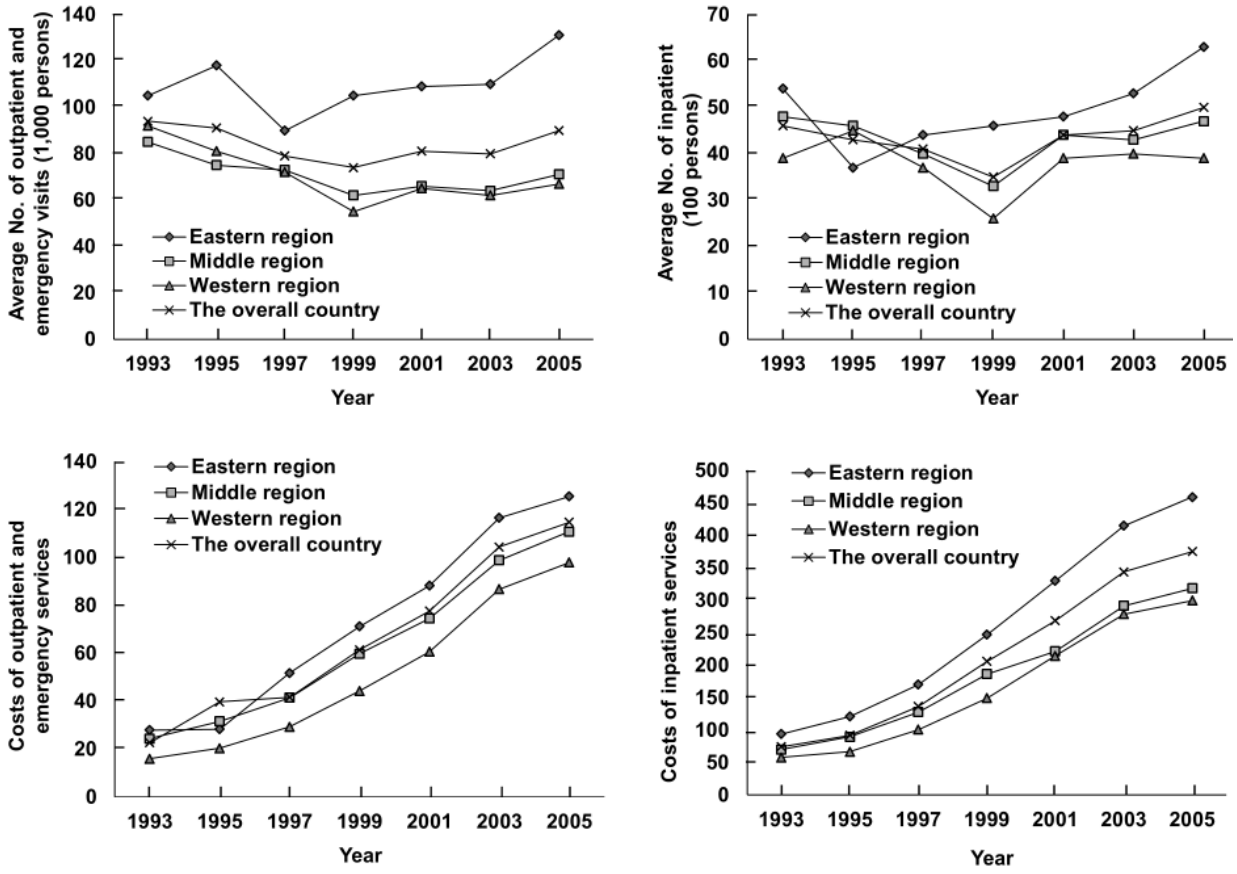

Figure 3. Amount and cost of health care services provided by county hospitals.

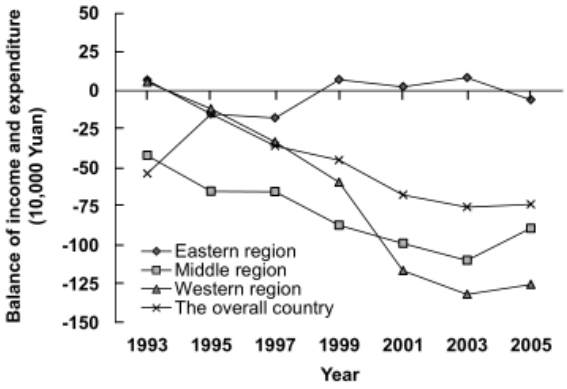

Figure 4. Balance of income and expenditures of county hospitals.

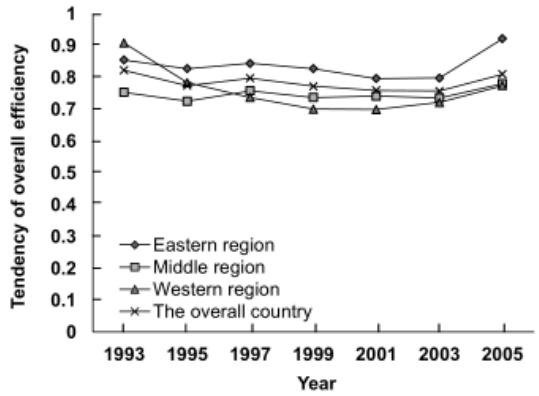

Figure 5. Overall efficiency based on DEA models.

efficiency of county hospitals decreased in comparison to 1993. In the eastern region, the average overall efficiency score increased from 0.854 in 1993 to 0.921 in 2005 and was higher than that in the middle and western regions. In the middle region, the score changed little. In the western region, however, it tended to decrease during the 12 years studied.

In 1993, county hospitals in 7 provinces (19.4%) were efficient although those in the remaining 25 provinces were inefficient. Among those provinces with inefficient county hospitals, four scored less than 70% and the score in the Tibet Autonomous Region was 0.505, the lowest. In contrast, in 2005 8 provinces (25.8%) reached the efficiency frontier and all of them were in the eastern region. The number of provinces with a score of less than 70% increased to eight. County hospitals in the Tibet Autonomous Region still had the worst efficiency, achieving only 46.6% of the frontier.

3.3.2. *Scale efficiency*

The score of scale efficiency was also measured by the C^2R model. In 1993, there were 4 provinces where county hospitals had decreasing returns to scale (14.9%), 7 provinces had constant returns to scale (22.6%), and 20 provinces had increasing returns to scale (64.5%). In 2005, 17 provinces had decreasing returns to scale (54.8%), 8 provinces had constant returns to scale (25.8%), and 6 provinces had increasing returns to scale (19.4%). Except for Hainan Province, provinces that had county hospitals with increasing returns to scale were located in the western region.

3.3.3. *Pure technical efficiency*

At the national level, in all years but 2003 pure technical efficiency reached the frontier. County hospitals in the eastern region have a higher pure technical efficiency score than those in the middle and western regions. Provinces that had county hospitals with pure technical efficiency were decreased by 9.7%, from 11 (35.5%) in 1993 to 8 (25.8%) in 2005.

3.3.4. *Input-output projection analysis*

An input-output projection analysis of those provinces having county hospitals with overall inefficiency in 2005 was also performed in order to examine over-supplied inputs and expected outputs by measuring the distance from them to their efficient peers. Results indicated that a reduction of 300,376 medical staff, a reduction of 310,975 beds, a reduction of 30,763,150,000 RMB of the value of fixed capital, or a reduction of 16,444,130,000 RMB in hospital expenditures will result in input savings at levels of 19.05%, 27.02%, 19.05%, and 19.05%, respectively. The distance to efficient practice also suggested the level of wasted health resources, which was 8% in the eastern region and more than 20% in the middle and western regions.

4. Discussion

This is the first study to examine the productive efficiency of all of China's county hospitals within the scope of the market-oriented health sector reform. From 1993 to 2005, the number of county hospitals increased and their inputs, *e.g.* fixed capital in particular, grew rapidly. Consequently, the costs of both outpatient and emergency services and inpatient services increased substantially. During the past two decades, county hospitals obtained autonomy with no public finances and were increasingly commercialized. Stimulated by market competition, they vastly enlarged their scale, including building extensions, bed installation, and the purchase of advanced equipment.

This expanded scale had both a positive and negative effect on the development of county hospitals and the accessibility of health care services. First, many county hospitals suffered severe deficits due to investment, worsening their financial situation. Second, the fact is that county hospitals enjoy few government subsidies, so their revenue mainly derives from drug costs and medical costs (*18*), and there are pervasive financial

incentives to over-provide services and over-use drugs in order to make a profit (*19-21*). Actually, like county hospitals, the enlarged scale and the financial incentives of all kinds of hospitals could be explained by soaring health expenditures, which are evident as a double-digit increase far exceeding the increase in individual income (*22*). Most rural residents paid out-of-pocket and were not covered by medical insurance, and rural cooperative medical schemes (CMS) in particular collapsed with the economic transition (*23-25*). Thus, increasing costs and fees of county hospitals resulted in a heavy burden for rural residents.

The rapid rise of medical and drug costs and lack of coverage by medical insurance for the rural population may have contributed to the decline in amounts of services provided by county hospitals, despite their expanded scale (*12,26*). The 2003 National Health Services Survey indicated that medical insurance covered only 21% of rural residents before CMS were re-established and that unaffordable medical costs were the main obstacle to access to health care services, particularly among rural residents (*22*). Beds were not being efficiently utilized. A previous survey agreed, indicating that the usage of large-scale advanced medical devices was less than 50% in many Chinese provinces (*27*). The poor utilization of newly invested inputs caused health resources to be wasted and impacted the return to scale, influencing overall efficiency.

This study revealed geographical disparities in health resource allocation and county hospital productivity, and gaps expanded during the 12 years studied. County hospitals in the eastern region tended to have slightly higher overall, scale and technical efficiency since their better socioeconomic status and financial capacity, larger population, and more convenient physical accessibility boosted the utilization of health care services there. The current findings also suggest considerable waste of health resources in the middle and western regions.

Based on the current findings, the dominant pattern in which Chinese county hospitals developed during the previous market-oriented reform of the health sector can be characterized as a significant expansion of scale along with commercialization. However, the amount of health care services these hospitals provided declined. The overall efficiency, return to scale, and pure technique efficiency of these hospitals decreased on the national level, and gaps in these indices among the eastern, middle, and western regions expanded. Such inefficiency of county hospitals, especially in the middle and western regions, should be addressed by the government-driven reform scheme, which has advocated a substantial increase in public investment, universal medical insurance coverage particularly for the rural population, restructuring of management systems, and corrections for increased commercialization in public hospitals (*10*). For most county hospitals with a decreased return to scale, regional health plans now emphasize improved utilization and quality of health care services in place of an expanded scale. In this regard, wide coverage by medical insurance with substantial risk pooling and containment of soaring medical expenditures will ensure the affordability and accessibility of health care services for the rural population (*28*). At their current scale, county hospitals are primarily tasked with developing human resources and improving medical skills and staff performance in order to optimize their management structures to effectively utilize inputs and improve the quality of health care services. Moreover, public financing is needed to close the expanded geographical gaps in health resource allocation and accessibility of health care services accompanying the profound impacts of decentralization. The effects of ongoing reform on the productivity of county hospitals must be monitored and evaluated to continue providing evidence for regional health plans.

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Original Article

Association between the serum folate levels and tea consumption during pregnancy

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Summary **Folate is a vital nutrient during pregnancy for the prevention of neural tube defects, intrauterine fetal growth restriction and preeclampsia. Circulating folate levels might be negatively affected by (–)-epigallocatechin gallate, which is a tea catechin found in green tea and oolong tea. The aim of this study was to determine whether consumption of green tea or oolong tea was associated with circulating folate levels among pregnant women in Japan. Two hundred and fifty-four healthy women with a singleton pregnancy (age: 30.4 ± 4.7, gestational age: 27.5 ± 9.6 weeks) were recruited from a prenatal clinic in metropolitan Tokyo, Japan. The serum folate levels were measured. Nutrient intake was assessed using a self-administered diet history questionnaire. Information on lifestyle variables was obtained from the questionnaire. The high consumption of green tea or oolong tea was defined as consumption more than 57.3 mL per 1,000 kcal, which is the 75th percentile of participants. The serum folate levels of the participants with high consumption of green tea or oolong tea was significantly lower than those of others ($p = 0.027$). A multiple regression analysis revealed the high consumption of green tea or oolong tea to be associated with a low serum folate level during pregnancy, after adjusting for confounding variables including dietary folate intake and use of folic acid supplements or multivitamins ($\beta = -0.131$, $p = 0.016$). The association between folate and the consumption of green tea or oolong tea may be useful to clarify the mechanism which links adverse perinatal outcomes and tea consumption.**

Keywords: Folate, catechin, tea, pregnancy

1. Introduction

Folate is a water-soluble B vitamin, which is absolutely essential for DNA synthesis, DNA repair and cell proliferation (*1*). Folate is required for the prevention of neural tube defects (NTDs), intrauterine fetal growth restriction (IUGR) and preeclampsia (*2-4*) before and throughout pregnancy. Therefore, the recommended intake of folate during pregnancy is 440 μg per day, which is almost twice as that of non-pregnant women (*5*). However, the average folate intake among Japanese pregnant women was about half of the recommended amount (*6-8*). In addition, the rate of pregnant women taking folic acid supplements was less than 30% (*6-8*), although the Ministry of Health, Labour and Welfare of Japan has recommended taking folic acid supplements for the prevention of NTDs since 2000 (*9*).The average serum folate levels of Japanese pregnant women were low in previous studies due to the decreased intake from diet or supplements (*6,8*), in comparison to that of pregnant women in other developed countries, where some foods are already fortified with folic acid, or supplements during pregnancy are in widespread use (*10-12*). Not only increasing folate intake but also giving up bad habits which interfere with the

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action of folate, such as smoking (*13*), is important for maximizing the effectiveness of the ingested folate, especially among such populations as that of Japanese pregnant women, who for the most part tend to consume insufficient amounts of folate.

Recently, (−)-epigallocatechin gallate (EGCG), which is a kind of tea catechin found in green tea and oolong tea (*14*), has been found to interfere in folate metabolism by inhibiting dihydrofolate reductase (DHFR) *in vitro* (*15,16*). DHFR is an important enzyme that reduces ingested folate to its active form, before folate is used in the body (*17*).

Most Japanese people consume green tea or oolong tea quite naturally at meals or with snacks. A previous study showed that about 50% of the Japanese pregnant women consumed green tea four or more times per day (*18*). Although the effect of EGCG on folate metabolism has been demonstrated *in vitro* (*15,16*), the relationship between folate status and daily consumption of tea among healthy pregnant or non-pregnant populations is unclear (*8,19,20*).

Therefore, the aim of this study was to determine whether consumption of green tea or oolong tea was associated with the circulating folate levels among pregnant women in Japan.

2. Materials and Methods

2.1. *Participants*

The present cross-sectional study was conducted at a private obstetric hospital from June to December of 2008 in metropolitan Tokyo, Japan. The study was approved by the Ethical Committee of the Graduate School of Medicine, at The University of Tokyo. Healthy women with a singleton pregnancy and no major complications such as diabetes or pregnancy-induced hypertension were recruited during their first trimester (8-12 weeks' gestation), their second trimester (24-27 weeks' gestation), or their third trimester (34-38 weeks' gestation) at a time when they were having routine blood tests. All the women underwent an ultrasound scan at 8-12 weeks' gestation to allow accurate gestational dating. The participants were given detailed information on the study protocol and all participants gave their written informed consent.

2.2. *Variables and their measurement*

Questionnaires were completed by each participant while waiting for a regular pregnancy checkup. Participants, who did not have sufficient time to complete the questionnaires at the hospital, filled out the questionnaires after returning home and then returned them by mail. All participants completed a questionnaire on their characteristics, including maternal age, gestational age, and pre-pregnancy body mass index (BMI). Information on lifestyle variables, such as smoking during pregnancy, the use of folic acid supplements or multivitamins including folic acid, and the frequency of supplement use (regular or irregular) was also obtained from the questionnaire. The regular use of folic acid supplements or multivitamins was defined as using such supplements four or more times per week.

The women's dietary intake over the last month was assessed with a validated self-administered diet history questionnaire (DHQ), which measures the daily intake of 150 foods and selected nutrients (*21,22*). Information on both the frequency and amount of consumption was collected. The folate intake and consumption of green tea or oolong tea were adjusted by energy to minimize the influence of dietary underreporting. Participants who had a severe under- or over-reported energy intake, namely, those whose reported energy intake was less than half the energy requirement for the lowest physical activity category, or those women whose reported energy intake was equal to or more than 1.5 times the energy requirement of moderate physical activity according to the "Dietary Reference Intakes for Japanese" (2005) were excluded from the present series (*5,23*).

Non-fasting blood samples were drawn at the clinic on the day of recruitment when the participants answered the questionnaires. Blood samples were centrifuged for 10 min at 3,000 rpm to separate the serum and then were stored at −80°C until the analyses were performed. The serum folate levels were measured using a chemiluminescent enzyme immunoassay (CLEIA). The assay was conducted by SRL, Inc., Tokyo, Japan.

2.3. *Statistical analysis*

The consumption of green tea or oolong tea was divided by the 75th percentile of participants into a high and low consumption group. The differences in characteristics between participants with high and low consumption of green tea or oolong tea were compared using the chi-square test, Student's *t*-test or the Mann-Whitney U test after normality was tested using the Shapiro-Wilk test.

A multiple regression analysis was used to determine whether consumption of green tea or oolong tea was associated with the serum folate levels. Dietary folate intake, the regular use of folic acid supplements or multivitamins, gestational age, pre-pregnancy BMI and smoking during pregnancy were adjusted as confounding variables. These variables were checked for multicollinearity. This multiple regression analysis was conducted after the logarithmic transformation of the serum folate levels because it showed skewed distribution.

Statistical analyses were carried out with the SPSS

software package for Windows, version 15.0 (SPSS Japan Inc.). All statistical tests were two-sided; a p value of less than 0.05 was considered to be statistically significant.

3. Results

A total of 321 pregnant women were recruited; of these, 292 (91.0%) gave their written informed consent, answered the questionnaire, and had blood samples drawn. Thirty-eight of the 292 participants were excluded from the analyses: 17 had missing data, 13 provided an inadequate amount of blood sample, 4 had pregnancy complications, and 4 had a severe under- or over-reported energy intake. The data from 254 pregnant women (79.1%) were analyzed; 38 were in the first trimester, 104 were in the second trimester and 112 were in the third trimester.

The characteristics of the participants are shown in Table 1. The 75th percentile of participants was used as the cutoff point for high consumption of green tea or oolong tea, 57.3 mL per 1,000 kcal. The cutoff point corresponded to consumption of 100-130 mL of tea per day.

Table 2 shows the median levels of serum folate, energy intake, folate intake and consumption of green tea or oolong tea, and the use of folic acid supplements or multivitamins. Some of the participants reported that they regularly consumed more than 1,000 mL of green tea or oolong tea per day. Serum folate levels among the participants with high consumption of green tea or oolong tea were significantly lower in comparison to others ($p = 0.027$). Meanwhile, the amount of black tea consumption, which contains only a small quantity of EGCG, was not associated with serum folate levels ($p = 0.704$).

There was a significant difference in the serum folate levels among each trimester (first trimester, 14.4 nmol/L; second trimester, 13.8 nmol/L; third trimester, 10.0 nmol/L; $p = 0.002$). No significant difference in the use of folic acid supplements or multivitamins was found among each trimester (first trimester, 28.9%; second trimester, 23.1%; third trimester, 16.1%; $p = 0.185$). In addition, there was no significant difference in the folate levels between smokers and non-smokers ($p = 0.184$).

Table 3 shows the relationship between the serum folate levels and the consumption of green tea or oolong tea. High consumption of green tea or oolong tea was significantly associated with a low serum folate level during pregnancy, after adjusting for confounding variables ($\beta = -0.131$, $p = 0.016$).

4. Discussion

This cross-sectional study found an association between low serum folate levels and high consumption of green tea or oolong tea during pregnancy, after various potential confounding variables, such as dietary folate intake and smoking during pregnancy, were taken into

Table 1. Characteristics of participants

Participants	All participants ($n = 254$)	Consumption of green tea or oolong tea < 57.3 mL/1,000 kcal ($n = 190$)	Consumption of green tea or oolong tea ≥ 57.3 mL/1,000 kcal[a] ($n = 64$)	p[b]
Age (years)	30.4 ± 4.7	30.5 ± 4.6	29.8 ± 5.0	ns
Gestational age				0.049
First trimester	38 (15)	25 (66)	13 (34)	
Second trimester	104 (41)	73 (70)	31 (30)	
Third trimester	112 (44)	92 (82)	20 (18)	
Height (cm)	157.9 ± 5.4	157.8 ± 5.4	158.3 ± 5.5	ns
Pre-pregnancy body mass index (kg/m^2)	20.8 ± 2.8	20.7 ± 2.6	21.2 ± 3.4	ns
Parity				ns
Primipara	136 (54)	104 (77)	32 (23)	
Multipara	118 (46)	86 (73)	32 (27)	
Smoking during pregnancy				ns
Smoker	8 (3)	7 (88)	1 (12)	
Non-smoker	246 (97)	183 (74)	63 (26)	
Education level				ns
High school and below	97 (38)	77 (79)	20 (21)	
Junior college/Technical college	106 (42)	73 (69)	33 (31)	
College/University	51 (20)	40 (78)	11 (22)	

Data are mean ± S.D. or n (%). ns: not significant. [a] The high consumption of green tea or oolong tea was defined as consumption more than 57.3 mL per 1,000 kcal, which is the 75th percentile of participants. [b] Student's t-test or the chi-square test was conducted.

Table 2. Biological marker, dietary intake and use of supplements

Items	All participants (n = 254)	Consumption of green tea or oolong tea < 57.3 mL/1,000 kcal (n = 190)	Consumption of green tea or oolong tea ≥ 57.3 mL/1,000 kcal[a] (n = 64)	p[b]
Biological marker				
Serum folate (nmol/L)	11.2 (8.6-19.5)	13.0 (8.8-20.9)	10.2 (8.2-14.4)	0.027
Dietary intake[c]				
Energy (kcal/day)	1,863 (1,614-2,135)	1,868 (1,615-2,142)	1,837 (1,601-2,109)	ns
Folate (μg/1,000 kcal)	110 (91-139)	108 (89-138)	111 (97-150)	ns
Consumption of green tea or oolong tea (mL/1,000 kcal)	11.5 (0.0-57.3)	0.0 (0.0-19.1)	138.8 (76.9-255.4)	0.017
Supplementation				
Regular use of folic acid supplements or multivitamins (more than 4 times/week)	53 (21)	41 (22)	12 (19)	ns

Data are median (interquartile range) or n (%). ns: not significant. [a] The high consumption of green tea or oolong tea was defined as consumption more than 57.3 mL per 1,000 kcal, which is the 75th percentile of participants. [b] The Mann-Whitney U test or the chi-square test was conducted. [c] The dietary intake was assessed with a validated self-administered diet history questionnaire (DHQ).

Table 3. Relationship between the serum folate levels and tea consumption during pregnancy

Items	All participants (n = 254)	
	β	p
Consumption of green tea or oolong tea (1: ≥ 57.3 mL/1,000 kcal, 0: < 57.3 mL/1,000 kcal)	−0.131	0.016
Dietary folate intake (μg/1,000 kcal)	0.110	0.043
Regular use of folic acid supplements or multivitamins (1: Yes, 0: No)	0.502	< 0.001
R^2	0.313	
Adjusted R^2	0.296	< 0.001

Multiple regression analysis was conducted, adjusting for gestational age, pre-pregnancy body mass index and smoking during pregnancy. The analysis was conducted after logarithmic transformation of the serum folate levels (nmol/L).

consideration.

Previous *in vitro* studies showed EGCG, which is an ester-bonded gallate catechin in green tea and oolong tea, to be an inhibitor of human DHFR activity (*15,16*). Folate drawn into the human body is absorbed from the small intestine. The absorbed folate is reduced by DHFR and changes into tetrahydrofolate (THF) (*17*). THF is methylated and changes into 5-methyl-THF (*17*). Folate is present in blood as 5-methyl-THF. Therefore, the inhibition of DHFR by EGCG decreases circulating folate levels. The current results might thus be explained by this mechanism.

Tea has been reported to have effects, such as anti-cancer, anti-oxidant effects, anti-inflammatory and promoting weight loss (*14,24-26*). This widely held positive belief may cause pregnant women to believe that tea is a healthy choice during pregnancy. In addition, advice by health care providers on tea consumption during pregnancy is inconsistent, even though teas have been generally acknowledged to contain caffeine that correlates with adverse pregnancy outcomes (*27*). Chu *et al*. showed that plasma EGCG levels in pregnant rats were about 1.5 times of those in non-pregnant rats when an equal amount of green tea was consumed (*28*). They have speculated that this difference may occur in association with changes in plasma protein composition and effective renal plasma flow. The folate status is likely affected by EGCG during pregnancy in comparison to non-pregnancy periods. No limits for the consumption of green tea or oolong tea can be established based on the results of the current study. However, health care providers might need to pay closer attention to the consumption of green tea or oolong tea in the future.

Correa *et al*. reported that maternal caffeinated tea consumption during the peri-conceptional period was associated with high rate of spina bifida, which is a kind of NTDs, although other caffeinated beverages were not associated with that risk (*29*). This result indicated that substance other than caffeine in tea, such as catechin, may affect the pathogenesis of NTDs. In the present study, consumption of green tea or oolong tea, which contains EGCG, was correlated with the level of serum folate, although black tea consumption, with little EGCG, did not correlate with serum folate levels. This result may suggest that substances in green tea or oolong tea, not black tea, are associated with folate status. Alternatively, because more than a half of the participants did not consume black tea, thus, the distribution of black tea consumption was more distorted than that of consumption of green tea or oolong tea, it might have been more difficult to show any statistical relationship between folate and black tea. Further studies are needed to investigate whether the circulating level of folate is affected differently by types of tea.

Folate has been identified as a nutrient required for fetal development, and prevention of NTDs and preeclampsia (*2-4*). In addition, folate plays a crucial role as an enzyme that prevents increasing homocysteine levels (*30-32*). A high tHcy level during

pregnancy is also a factor contributing to adverse perinatal outcomes, including NTDs, preterm birth, placental abruption, stillbirth and preeclampsia (*33-35*). Therefore, preventing low folate levels during pregnancy is important for both pregnant women and their fetuses. However, the folate intake among 75% of the current participants was far below 440 μg per day, which is the recommended intake for pregnant women (*5*). In addition, the rate of the participants taking folic acid supplements or multivitamins was only 21%, which was far lower than that other developed countries (*11,12,36*). Pregnant women need to be advised to take a sufficient amount of folate from dietary or take folic acid supplements in order to increase the circulating folate levels, as well as to cut down on excessive consumption of either green tea or oolong tea.

This study had several limitations. First, the number of participants was small, and this may have reduced the overall statistical power. Second, the effect of green tea or oolong tea on the serum folate levels could not be independently clarified, because the participants tea consumption was asked as a single question. Third, the questionnaire did not indicate the types of green tea or oolong tea and the brewing method, although the catechin content of tea varies depending on the types and the extraction time. Therefore, the results presented here should be examined by larger studies that include more detailed information on the types and brewing methods of various types of tea.

Despite these limitations, this study has useful clinical implications. A high consumption of green tea or oolong tea was associated with low serum folate levels among healthy pregnant women, after adjusting for several confounding variables. The association between folate and consumption of either green tea or oolong tea may therefore be useful for elucidating the mechanism which links tea consumption and adverse perinatal outcomes.

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Original Article

Nurse risk managers' criteria for dealing with near-miss events

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Summary **From the preventive point of view, it is very valuable for Japanese hospital safety managers to select important cases not only from among accident events, but also from near-miss events that involve errors that may result in the occurrence of future serious adverse events. The objective of this study is to investigate factors that determine the type of analysis that applies to hypothetical near-miss events. We sent self-administered questionnaires to 393 nurse risk managers from general hospitals in Japan. Hypothetical near-miss events were presented, and respondents assessed hypothetical events. Type of Analysis, Probability, Organizational Risk (effect on reputation and effect on cost), and Severity (possibility of harm, degree of harm, possibility of recovery, and possibility of delayed discharge) were included in the questionnaire. Response rate was 47.3% (186/393) and finally 175 nurses are analyzed. The respondents were 58 full-time safety managers (33.1%) and 117 who were safety managers concurrently with other work (66.9%). As a result of logistic regression analysis, probability, effect on reputation, possibility of harm and possibility of delayed discharge were significant ($p < 0.05$). Japanese safety managers consider near-miss events that have a lower probability to be more important. This finding differs from existing prioritization systems that were principally made for actual adverse events. It may suggest the problem of uncritically applying scales for accident events to near-miss events.**

Keywords: Patient safety, risk management, adverse event, near-miss, incident report

1. Introduction

An incident reporting system is a risk assessment tool in which the health care staff voluntarily reports adverse events that occurred in their work places. This assessment tool is convenient, and many health care facilities use it. It is said that incident reporting systems cannot provide the true incidence of adverse events (*1,2*). However, such a system is able to gather data not only on incidents as actual adverse events (errors that were not prevented) – which is called an accident – but also on incidents as potential adverse events (errors that were prevented by planned or unplanned barriers) – which is called a near-miss. Although the difference between them is usually unclear in a reporting system, considering the nature of near-miss events that were prevented, an analysis focusing on near-miss events plays an important role in preventing actual adverse events (*3,4*).

In many health care facilities, when the staff discovers an accident or a near-miss, he or she reports it to the appropriate supervisor (*e.g.* risk manager). The supervisor records the reported incidents, analyzes them using descriptive statistics, and informs staff of the results of analysis. Particularly important cases are analyzed in detail to identify the cause and corrective action is implemented (we call this detailed analysis an expanded analysis). From the preventive point of view, an expanded analysis is one of the most important stages in safety management activities to reduce adverse events systematically. However, except for some facilities in the U.S., there is no common methodology as to how safety managers can prioritize the huge number of reports of adverse events to select important cases for expanded analysis. Some facilities

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in the U.S. have developed criteria and an algorithm to select events that require close attention (*5*).

The Japanese health care systems is increasingly recognizing the importance of medical safety managers, and in 2002 national hospitals were ordered to assign a full-time risk manager to each facility. But many safety managers perform safety management activities concurrently with their usual work as health care providers. Because they cannot spend a large amount of time on safety management activities, they tend to select more accident events than near-miss events for expanded analysis, especially when they are newly appointed or busy. But as described above, from the preventive point of view, it is very valuable for safety managers to select for close scrutiny important cases not only from among accident events, but also from near-miss events that involve errors that may result in the occurrence of future serious adverse events.

In view of these considerations, it would be quite useful for future safety managers in health care facilities to know how present safety managers select important cases for expanded analysis. In this study, we developed questionnaires based on prioritization systems that were developed by two health care facilities in the U.S. and sent them to nurses appointed as risk managers, asking which type of analysis they apply to hypothetical near-miss events. The objective of this study is to investigate factors that determine the type of analysis applied to near-miss events.

2. Methods

2.1. *Participants and study design*

Participants were 393 general hospitals in Japan listed on "Byoin-Yoran 2001-2002 (Japanese hospital directory 2001-2002)" (*6*) that have 500 or more beds. Not included were hospitals in which half or more beds are for long-term or psychiatric care. We sent a self-administered questionnaire by post to each of these hospitals and requested one of the nurses appointed as safety manager to answer the questionnaire.

2.2. *Development of items*

We referred to the Risk Assessment Index (RAI) of the Medical Event Reporting System for Transfusion Medicine (MERS-TM) (*3,7*) and the Safety Assessment Code (SAC) used in the Veterans Health Administration (VHA) to develop the items on the questionnaire (*8*). RAI and SAC are scales for scoring adverse events to determine the type of analysis and way of handling the event.

Both scales assess events from the viewpoint of severity of harm to the victim, probability of recurrence in the facility, and organizational risk (*e.g.* damaged reputation or financial loss) and generate a score for each event. When scores are higher there is a greater need for an expanded analysis. To assess the severity of a near-miss event, RAI considers potential harm, while SAC considers the most likely "worst case".

In the questionnaire that we developed, first we described hypothetical near-miss events, and then, with regard to each hypothetical case, asked about the probability of occurrence of such a near-miss event in their facility, organizational risk, and type of analysis that they would select for the event if it had actually occurred in their facility. Furthermore, we asked about consequences to the victim if the hypothetical near-miss event were not prevented. Specifically, the questionnaire consisted of four subsections: Probability, Type of Analysis, Organizational Risk, and Severity.

The Probability subsection consists of one item, with 4 possible responses (a, several times or more per month; b, several times within per half year; c, several times per year; d, once or less per year). The Type of Analysis subsection also has one item but with 5 possible responses (a, expanded analysis; b, investigation to find common problems; c, descriptive data analysis; d, no analysis; e, other type of analysis). The Organizational Risk subsection consisted of two items, one on the effect on the institution's reputation and the other on costs; each could be answered by: a, large effect; b, small effect; or c, no effect. The Severity subsection included 4 possible scenarios. The first was the possibility of harm (a, almost certain; b, highly possible; c, rare); the second was possible degree of harm (a, fatal; b, Activities of Daily Living (ADL) disability highly possible; c, ADL disability only slightly possible); the third is the possibility of recovery from the resultant harm (a, no recovery highly possible; b, recovery highly possible), and the fourth is the possibility of delayed discharge (a, highly possible; b, only slightly possible). The 7 items on the Probability, Organizational Risk, and Severity subsections were factors that determined the type of analysis for a near-miss event.

Possibility of harm was based on the RAI. Possible degree of harm, possibility of recovery, and possibility of delayed discharge were based on severity categories of the SAI that include concrete descriptions of injuries that the victim could suffer.

2.3. *Development of hypothetical near-miss events*

To develop hypothetical near-miss events, we referred to the 2nd Summary and Tabulation of Network Maintenance Projects for Medical Safety (project including incident collection) by the Ministry of Health, Labor and Welfare (*9*). First, we selected some incidents from the released summary and tabulation to satisfy the following: Description objective, Description detailed, Incident nurse discovered, and Prevention possible in the interval between occurrence of error and actual or

possible harm to patient.

Incidents in the released summary and tabulation include many events in which errors were not prevented, because the definition of the incident is not identical with that of the near-miss event in this study. Therefore, we converted those incidents into near-miss events with minimal adjustments and developed 10 hypothetical near-miss events. From several discussions with 11 nurses with 5 to 19 years of nursing experience, we removed one hypothetical near-miss event. Finally, we used 9 hypothetical near-miss events in our questionnaire.

Characteristics of events are based on the results of interviews with several risk managers in Japan, and consist of 7 items as shown in Table 1. The 7 items are: type of event with regard to whether it was related to medication or other causes; whether event was discovered by a planned barrier or by chance; whether or not the error was discovered near the patient or far from the patient; whether only a nurse is related between the error and discovery or many occupations participated; if the discoverer was the nurse who made the error, a colleague or a patient; if the patient contributed to the error, and, finally, if a nurse was responsible for the error or the other occupations were responsible.

Table 1. Characteristics of hypothetical near-miss events

Event number	1	2	3	4	5	6	7	8	9
Type of event									
Medication related	●	●	●	●					
Others					●	●	●	●	●
Cause of discovery									
Planned barrier	●				●	●	●		
Chance		●	●	●				●	●
Place of discovery									
Far from the patient	●	●							
Near the patient			●	●	●	●	●	●	●
Related occupation									
Only nurses				●			●	●	●
Many occupations	●	●	●		●	●			
Discoverer									
Nurse making the error			●		●	●	●		●
Colleague	●	●						●	
Patient				●					
Patient contribution									
Did not contribute	●	●	●	●	●				●
Contributed						●	●	●	
Responsibility									
Nurse	●	●		●		●	●	●	●
Other occupation			●		●				

2.4. *Development of questionnaire*

To reduce the burden of the participants, we randomly ordered the 9 hypothetical near-miss events and used five events (events 1 to 5) in the first questionnaire, five events (events 2 to 6) in the second questionnaire, and so on, repeating the process nine times. The result was 9 different questionnaires sent to participants at random.

2.5. *Statistical analysis*

We converted answers from participants to Type of Analysis into a categorical variable with three levels (expanded analysis, investigation to find common problems (common problem investigation), and others). We used chi-square tests to examine the relations between Type of Analysis and determinant factors (Probability, Organizational Risk (2 items), and Severity (4 items), the characteristics of participants and characteristics of events). Length of appointment and length of time reporting system that was implemented were converted into dichotomous variables by considering their distributions.

We used logistic regression analysis with the forced entry method to investigate factors that determined the type of analysis applied to a near-miss event, using dummy variables for 9 types of events to adjust for the effect of differences among these events. As the dependent variable, we used Type of Analysis with the three levels described above and dichotomous variables (expanded analysis and others), implementing multiple logistic regression analysis and binary logistic regression analysis, respectively. As explanatory variables, we selected the variable from the determinant factors that had *p*-values on chi-square tests less than 0.1.

These statistical analyses were implemented for all the participants (total group), participants who were full-time safety managers (full-time group), and participants who were safety managers concurrent with other work (concurrent group), respectively.

We analyzed the data using the statistical package SPSS 10.1J for Windows, and used a 5% significance level.

3. Results

3.1. *Characteristics of participants*

Responses to our request for participation were received from 186 of the nurses appointed as safety managers (response rate, 47.3%; number of hypothetical near-miss events, 930). Excluded from participation were nurses who left any question on characteristics of participants unanswered. Similarly, when a question about an event was missing, that event was excluded from analysis. As a result, 175 nurses participated in this study and 808 hypothetical near-miss events were answered.

Characteristics of participants are shown in Table 2. There were 58 full-time safety managers (33.1%). For statistical analysis, length of appointment was divided into less than 2.5 years and 2.5 years or more, and length of time reporting system that was implemented was

Table 2. Characteristics of participants

Participants	Frequency (%)/Mean (S.D.)					
	Total (n = 175)		Full-time (n = 58)		Concurrent (n = 117)	
Sex						
Male	6	(3.4)	1	(1.7)	5	(4.3)
Female	169	(96.6)	57	(98.3)	112	(95.7)
Age	50.0	(5.1)	48.6	(5.1)	50.7	(5.0)
Length of employment	20.8	(10.3)	15.7	(10.9)	23.4	(8.9)
Length of appointment	2.3	(2.8)	1.1	(1.1)	2.9	(3.1)
Emplyment status						
Vice director of nursing division or higher	58	(33.1)	5	(8.6)	53	(45.3)
Others	117	(66.9)	53	(91.4)	64	(54.7)
Appointment form						
Full-time	58	(33.1)				
Concurrently with other work	117	(66.9)				
Area from which reports are submitted						
Including other division	82	(46.9)	50	(86.2)	32	(27.4)
Nursing division only	93	(53.1)	8	(13.8)	85	(72.6)
Authority						
Review of related documents	139	(79.4)	53	(91.4)	86	(73.5)
Interview with staff	108	(61.7)	52	(89.7)	56	(47.9)
Interview with patient	53	(30.3)	27	(46.6)	26	(22.2)
Administration of staff education	139	(79.4)	52	(89.7)	87	(74.4)
Development or revision of manuals	135	(77.1)	45	(77.6)	90	(76.9)
Determination of instruments and materials used	72	(41.1)	26	(44.8)	46	(39.3)
Recommendation for preventing adverse events	136	(77.7)	49	(84.5)	87	(74.4)
Announcement of information about events	104	(59.4)	50	(86.2)	54	(46.2)
Holding the risk management committee meeting	75	(42.9)	32	(55.2)	43	(36.8)
Number of beds						
~ 599	82	(46.9)	24	(41.4)	58	(49.6)
600 ~ 699	39	(22.3)	16	(27.6)	23	(19.7)
700 ~ 799	26	(14.9)	12	(20.7)	14	(12.0)
800 ~	28	(16.0)	6	(10.3)	22	(18.8)
Length of time reporting system implemented	7.6	(6.5)	6.6	(6.6)	8.1	(6.5)

divided into less than 5 years and 5 years or more.

3.2. *Determinant factors of Type of Analysis (all participants)*

3.2.1. *Relations between Type of Analysis and individual determinant factors*

We examined the relations between Type of Analysis and individual determinant factors (Table 3). All of the factors had a significant relationship with Type of Analysis ($p < 0.05$). Moreover, 3 items under characteristics of events (place of discovery, patient contribution, and responsibility) had a significant relationship with Type of Analysis ($p < 0.01$).

3.2.2. *Determinant factors of Type of Analysis*

We implemented a multiple logistic regression analysis using Type of Analysis as the dependent variable with three levels that consisted of "expanded analysis", "common problem investigation", and "others". We used "others" as a reference category. As a result of forced entry of determinant factors and dummy variables for events (Cox and Snell R square = 0.243, Nagelkerke R square = 0.283, MacFadden R square = 0.142), the factors that made the safety managers implement expanded analysis were: low probability (once or less per year, $p < 0.01$; several times per year, $p < 0.05$; several times pre half year, $p < 0.05$), effect on reputation (large effect, $p < 0.001$), high possibility of harm (almost certain, $p < 0.05$), and appointment form (full-time safety manager, $p < 0.01$). Factors that compelled the safety managers to implement common problem investigations were: only a large effect on reputation (large effect, $p < 0.001$) and high degree of harm (high possibility of ADL disability, $p < 0.01$).

Next, we implemented a binary logistic regression

Table 3. Relations between Type of Analysis and individual determinant factors

Individual determinant factors	*n*	Expanded analysis	Common problem investigation	Others
Probability***				
Once or less a year	234	78%	12%	10%
Several times a year	242	50%	31%	19%
Several times half a year	206	49%	33%	19%
Several times or more a month	126	37%	34%	29%
Effect on reputation***				
Large effect	217	70%	22%	8%
Small effect	330	50%	29%	21%
No effect	261	47%	27%	25%
Effect on cost**				
Large effect	178	65%	23%	12%
Small effect	377	52%	29%	19%
No effect	253	51%	26%	23%
Possibility of harm***				
Almost certain	144	70%	18%	12%
Highly possible	331	50%	33%	18%
Rare	333	38%	33%	30%
Degree of harm***				
Fatal	242	69%	17%	14%
ADL disability highly possilble	306	52%	34%	14%
ADL disability only slightly possible	260	47%	28%	26%
Possibility of recovery*				
No recovery highly possible	572	63%	21%	16%
Recovery highly possible	236	53%	29%	19%
Possibility of delayed discharge***				
Highly possible	352	62%	23%	15%
Only slightly possible	456	48%	31%	21%
Appointment form**				
Full-time	266	60%	28%	12%
Concurrently with other work	542	54%	26%	21%
Length of time reporting system implemented*				
≥ 5 years	396	59%	22%	19%
< 5 years	412	52%	31%	17%
Length of appointment*				
≥ 2.5 years	299	53%	24%	23%
< 2.5 years	509	57%	28%	15%

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

analysis using Type of Analysis as the dichotomous dependent variable that consisted of "expanded analysis" and "others". The result of this analysis is shown in Table 4 (dummy variables for the events are not on this table). Probability (once or less per year, $p < 0.001$; several times per year, $p < 0.05$), effect on reputation (large effect, $p < 0.001$), possibility of harm (almost certain, $p < 0.05$), and possibility of delayed discharge (high possibility, $p < 0.05$) had significant effects. Regarding Probability, the lower the chance of probability, the higher the odds ratio, which means there was an increased tendency to select for expanded analysis. Appointment form ($p < 0.01$) and length of reporting ($p < 0.01$) were also significant, suggesting that full-time safety managers and those in a facility with a reporting system in place for 5 years or more analyzed near-miss events in detail.

3.3. *Determinant factors of Type of Analysis (full-time group)*

Of the participants, 58 were full-time safety managers (Table 2) and these participants provided information for 266 hypothetical near-miss events.

The result of chi-square tests suggested that Type of Analysis for the full-time group had a significant relationship with all items within Probability, Organizational Risk, and Severity ($p < 0.01$), but not with length of appointment and length of time the reporting system was implemented. About the relationship between Type of Analysis and characteristics of events, place of discovery, related

Table 4. Binary logistic regression analysis

Individual determinant factors	Total (n = 808)	Full-time (n = 266)	Concurrent (n = 542)
	OR	OR	OR
Probability			
Once or less a year	4.267***	13.672***	3.124**
Several times a year	1.886*	3.034*	1.560
Several times half a year	1.563	1.497	1.604
Several times or more a month			
Effect on reputation			
Large effect	2.831***	1.131	1.199
Small effect	1.232	2.357	3.117***
No effect			
Effect on cost			
Large effect	0.716	0.560	1.144
Small effect	0.859	0.855	0.682
No effect			
Possibility of harm			
Almost certain	2.243*	2.288	0.893
Highly possible	1.362	2.278	1.835
Rare			
Degree of harm			
Fatal	0.842	0.633	0.819
ADL disability highly possilble	0.778	1.336	0.787
ADL disability only slightly possible			
Possibility of recovery			
No recovery highly possible	0.807	0.507	0.790
Recovery highly possible			
Possibility of delayed discharge			
Highly possible	1.650*	4.888**	1.249
Only slightly possible			
Appointment form			
Full-time	1.688**		
Concurrently with other work			
Length of time reporting system implemented			
≥ 5 years	1.713**	1.451	1.835**
< 5 years			
Length of appointment			
≥ 2.5 years	0.974	0.272*	1.127
< 2.5 years			
Cox & Snell R^2	0.196	0.318	0.178
Nagelkerke R^2	0.263	0.431	0.238

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

occupation, patient contribution, and responsibility were significant ($p < 0.05$).

We implemented a binary logistic regression analysis for the full-time group using Type of Analysis as the dichotomous dependent variable. As shown in Table 4, items within Probability (once or less per year, $p < 0.001$; several times per year, $p < 0.05$) and possibility of delayed discharge (high possibility, $p < 0.01$) had significant effects.

3.4. *Determinant factors of Type of Analysis (concurrent group)*

We equally implemented statistical analysis for safety managers who assumed that role concurrently with other duties. In this category were 117 participants (Table 2) and 542 hypothetical near-miss events.

As a result of chi-square tests, 6 items (probability, effect on reputation, possibility of harm, degree of harm, possibility of delayed discharge, and length of implementation of a reporting system) had a significant relationship to the Type of Analysis with the concurrent group ($p < 0.05$). In the test of the relationship between Type of Analysis and characteristics of events, significance was identified for place of discovery, patient contribution, and responsibility ($p < 0.01$).

As to the binary logistic regression analysis for the concurrent group using Type of Analysis as the dichotomous dependent variable, as shown in Table 4, Probability (once or less per year, $p < 0.01$), effect

on reputation (large effect, $p < 0.001$), and length of implementation of reporting system ($p < 0.01$) had significant effects.

4. Discussion

4.1. *Characteristics of participants*

Because there are few studies on safety managers in Japanese health care facilities, we have little information to make comparisons regarding the characteristics of subjects of our studies with those of other safety managers. Length of appointment of our participants was relatively short (mean; 2.3 years), suggesting that many facilities had only recently established a safety management section or that the term for a safety manager is relatively short. Of the respondents, 33.1% served as vice director of the nursing division or had some other upper-level position. In this study, we requested one of the nurses who was appointed as safety manager to answer the questionnaire, so administrative staff such as a so-called general risk manager tended to answer the questionnaire. We must keep in mind that the administrative view of the participant might affect responses.

That 33.1% of the respondents were full-time safety managers supports results of research of the Japanese Nursing Association in 2001 that approximately 60% of health care facilities with risk managers do not have a full-time risk manager.

4.2. *Characteristics of events*

We examined the validity of hypothetical near-miss events in this study. Seven items under "characteristics of events" were significantly associated with 4 items of Type of Analysis for the total group, 3 items for the full-time group, and 3 items for the concurrent group. Three of the items were identical for each group except for 1 item in the total group (discoverer). Safety managers in all groups selected more detailed analysis for events that were discovered far from the patient, to which the patient contributed, and for which a nurse was responsible.

The results with regard to both patient contribution and responsibility were as would be expected. Events that were discovered far from the patient were related to important medications such as noradrenalin or insulin in this study, so a relation between the place of discovery and detailed analysis can also be expected. There was consistency between characteristics of events and selection of Type of Analysis, and therefore the hypothetical near-miss events in this study are valid.

4.3. *Determinant factors of Type of Analysis*

We referred to American scales for determination of Type of Analysis and treatment to develop items in our questionnaire. The results suggest that Japanese safety managers also use potential severity to assess near-miss events (errors that were prevented by planned or unplanned barriers). The higher the potential severity, the more frequently expanded analysis is selected for the event.

There is a difference in regard to Type of Analysis with Probability. Both SAC and RAI select more expanded analysis for events with higher probability (*3,10*). But in this study, safety managers tended to select more frequently expanded analysis for events of lower probability. The results of logistic regression analysis also support this finding.

This difference might depend on the type of event. While SAC and RAI deal with both accident events and near-miss events, the events in this study are all near-miss events. Because a patient can suffer harm from an actual adverse event, the higher the Probability of the event, the more important the event is. Many papers support this relationship (*11-13*). By contrast, although there are many articles that suggest the importance of a near-miss event, there are few articles about the relationship between Probability and importance of near-miss events (*14,15*). In this study safety managers consistently selected more expanded analysis for events of lower Probability. These findings suggest the problem that the scales principally made for actual adverse events were uncritically applied to near-miss events.

The results of logistic regression analysis for the total group shows that 1 item of Organizational Risk (effect on reputation) and 2 items on Severity (possibility of harm and possibility of delayed discharge) are significant. In this study, non-significant items for Severity (degree of harm and possibility of recovery) require a relatively vivid imagination about a patient's injury and its results, and also the effect on cost. It is difficult for safety managers to have such a clear imagination. In contrast, significant items require relatively little imagination about the event. Thus, it is easier for safety managers to make predictions regarding significant items than non-significant ones. The hypothetical events used in this study provide no information about the individual patient. In an actual near-miss event, expanded patient information might affect the selection, especially prediction of Severity.

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Original Article

Classification of hard core and petty criminals using anthropometric measurements

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Summary **The purpose of the present study was to compare the somatometric measurements among hard core criminals, petty criminals and community people. Using standard anthropometric procedures, somatometric dimensions were studied on 250 subjects each from the three groups: (*i*) experimental (hard core criminal) group, (*ii*) control-I (petty criminal) group, and (*iii*) control-II (community people) group. Univariate analysis of variance was used for making comparisons of somatometric measurements between these groups in the univariate case. Quadratic discriminant analysis (QDA) was used to develop a model based on measurements that classifies the cases into groups. The study revealed that the somatometric measurements such as morphological total facial height ($p < 0.01$), physiognomic total facial height ($p = 0.015$), nasal length ($p = 0.001$), height of lower face ($p = 0.001$), nasal depth ($p = 0.002$), sitting height vertex ($p = 0.011$), bigonial breadth ($p < 0.001$), maximum head breadth ($p = 0.001$), morphological upper facial height ($p < 0.001$), and physiognomic ear breadth ($p = 0.039$) were significantly different between the three groups. Morphological upper facial height, physiognomic total facial height, nasal length and height of lower face could be used as identifying factors for hard core criminals. Morphological total facial height, physiognomic upper facial height, physiognomic ear breadth, and sitting height were found different significantly in the experimental group as compared to the control-II group. The QDA provided an overall 72.4% correct classification of cases and 74.5%, 69.6%, and 72.9% correct classification for the experimental, control-I and control-II groups, respectively. The blurred distinction of the three groups could be explained by using the QDA model.**

Keywords: Somatometric measurements, hard core criminals, petty criminals, Kruskal-Wallis test

1. Introduction

The common belief that body-build is somehow related to function not only in general behavior, temperament, and disease as well as in socially unacceptable acts finds expression in folk-saying, verse, clinical observation, *etc*.

In a system of identifying criminals, the first use of anthropometrics was designed in the late 19th century by Alphonse Bertillon, a French criminologist. A study was done to find out a relationship between physique and intelligence of the criminals by Mohr and Gundlach (*1*). A small negative correlation between physique and intelligence of criminals was found by them. An anthropometric study of body-build among Illinois male prisoners was made by Gray (*2*). A major study of crime and morphology was done by Hooton on native white criminals of nine states in the United States (*3-5*). The anthropometric measurements on 4,000 males obtained by Snodgrasse revealed a very high correlation between physic and temperament of persons (*6*). A study to relate biological variables to criminal behavior was done by Ellis (*7*). Genovese (*8*) found a correlation between anthropometric measurements and IQ of criminals. Pavlich (*9*) analyzed two techniques, Alphonse Bertillon's techniques for measuring bodies

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and Francis Galton's composite portraits of criminal types, for identification of criminals.

The aim of the present study was to explore the extent to which the constitution, especially body build, and other morphological traits cause minor as well as major crime tendencies. For this purpose, we have selected three groups: (*i*) experimental (hard core criminal), (*ii*) control-I (petty criminal), and (*iii*) control-II (community people) in Uttar Pradesh, a northern state of India. Uttar Pradesh (area 240,928 sq. km.) is the largest state of India, consisting of about a 166.05 million population. In this study, 24 different somatometric dimensions were studied on 250 subjects from each of the three groups. Most of the studies in this area had used one way analysis of variance for comparison of somatometric dimensions of the study groups. In the present study discriminant analysis (DA) was used for the first time in order to make meaningful and substantive conclusions.

2. Methods

2.1. *Subjects*

The sample for the present study consisted of 250 subjects each from experimental, control-I, and control-II groups. Experimental and control-I inmates were selected from prisons of the widely spread five districts (Lucknow, Barabanki, Kanpur, Unnao, and Sitapur) covering more than one-third of the population of Uttar Pradesh.

2.2. *Definitions*

2.2.1. *Experimental (hard core criminal) group*

Inmates charge-sheeted for major offences (murder, attempt to murder, kidnapping, rape, forgery, robbery, dacoit, and gangster) under specified criminal sections at least two times on different occasions with their cases being admitted by the courts of law for trial.

2.2.2. *Control-I (petty criminal) group*

Prisoners charge-sheeted for less serious offences (theft, house breaking, bribery, dishonestly receiving stolen property, hurt, rash driving, journey without ticket, gambling, and keeping arms unlawfully) under specified sections at least two times on different occasions with their cases being admitted by the courts of law for trial.

2.2.3. *Control-II (community people) group*

Neighbors of subjects of the experimental groups belonging to the same age-group and socio-demographic background, having no evidence of specified criminal behavior and willing to cooperate.

2.3. *Measuring instruments*

In somatometry, several instruments were used to take various measurements of different parts of the body. However, the most common instruments used for measurements were the spreading calliper, sliding calliper, anthropometer, rod compass, and measuring tape.

2.4. *Measurements*

Most of the measurements were taken from one landmark to another. Specific precautions were taken to know the definition of the landmark, to locate them accurately and then to take measurements correctly with the help of following a standard technique and instruments.

Measurements were taken on the 24 somatometric dimensions of the subjects. These dimensions are maximum head length (MAHL), maximum head breadth (MAHB), maximum head height (MAHH), minimum frontal breadth (MFRB), bizygomatic breadth (BIZB), bigonial breadth (BIGB), morphological upper facial height (MUFH), morphological total facial height (MTFH), physiognomic total facial height (PTFH), physiognomic upper facial height (PUFH), nasal length (NASL), nasal breadth (NASB), nasal depth (NASD), mouth breadth (MOUB), height of the lower face (HELO), physiognomic ear length (PEAL), physiognomic ear breadth (PEAB), height vertex or stature (HVER), height acromion(HACR), height iiliocristale (HILI), sitting height vertex (SHVE), biacronial breadth (BIAB), bicristal breadth (BICB), and transverse chest breadth (TCHB). All the measurements were taken in centimeters (cm).

2.5. *Statistical analysis*

For the comparison of somatometric measures between the three groups the univariate analysis of variance (ANOVA) technique was used. For making multiple comparisons Tukey's test was used. In paired comparison, two-tailed tests were implied. Discriminant analysis was used to develop a model based on measurements that classify the cases into different groups. This model can be used for the classification of the additional observations into correct groups. Several methods are defined for the discriminant analysis from which linear discriminant analysis (LDA) and quadratic discriminant analysis (QDA) are important methods. When the covariance matrices for each group were the same, linear discriminant analysis was used, otherwise quadratic discriminant analysis was used. Measurements found significant (or nearly significant, $p \leq 0.1$) in ANOVA were used for the discriminant analysis. The whole analysis was done using software R-2.7.0.

3. Results

The three groups were comparable across socio-demographic characteristics (Table 1). The majority of the persons in each group were male (above 95%). The majority of persons (nearly 80% in each group) were below 33 years of age. A little more than half of the persons in each group were inhabitants of an urban area (52%) and nearly one-third from each group were employed in a farming occupation. Nearly half of the subjects in each group were either illiterate or had education below high school. In the control-II groups, the number of subjects married in the experimental group (51.6%) was less compared to community people (64%).

The somatometric measurements between groups were compared by using univariate comparisons (ANOVA). The main assumption of ANOVA is that the measurements should be normally distributed. By using the Shapiro-Wilk's statistic, it was observed that measurements were not normally distributed. The Kruskal-Wallis test (non-parametric analog of ANOVA) was used for univariate comparisons because the assumption of normality failed. The results of univariate comparisons are shown in Table 2. The measurements MAHB, BIGB, MUFH, MTFH, PTFH, PUFH, NASD, NASL, PEAB, PEAL, HVER, HACR, SHVE, and HELO were significantly different between the groups.

Because the assumption of normality failed pair-wise comparisons were done, using the Mann-Whitney *U*-Test, for measurements found significant ($p < 0.05$) in univariate comparisons. The results of pair-wise comparisons are presented in Table 3. The measurements MUFH, MTFH, PUFH, NASL, HELO, HACR, and SHVE were significantly different between the experimental group and control-I group as well as control-II group. The measurements MAHB, PTFH, NASD, PEAL, and HVER were significantly different between the experimental group and control-I group, whereas BIGB and PEAB were different between the experimental group and control-II group. MAHB, BIGB, PUFH, NASD, and PEAL were significantly different between the control-I group and control-II group.

The homogeneity of covariance matrix of measurements was tested using Box's *M*-test because it is an important assumption for LDA. The covariance matrices for the three groups were found significantly different ($p < 0.001$). Because the data was not normally distributed, robust quadratic discriminant analysis was used for fitting the classification model. The classification obtained by robust LDA and robust QDA were compared using the original measurements. An overall correct classification of 72.4% was obtained by robust QDA (Table 4).

4. Discussion

The study of somatometric associates of aberrant

Table 1. Socio demographic profile of each group

Variables	Subject type		
	Experimental	Control-I	Control-II
Sex			
Male	239 (95.6%)	239 (95.6%)	238 (95.2%)
Female	11 (4.4%)	11 (4.4%)	12 (4.8%)
Age			
Less than 33 years	200 (80.0%)	205 (82.0%)	197 (78.8%)
Between 33-43 years	44 (17.6%)	36 (14.4%)	50 (20.0%)
More than 43 years	6 (2.4%)	9 (3.6%)	3 (1.2%)
Domecile			
Rural	120 (48.0%)	120 (48.0%)	120 (48.0%)
Urban	130 (52.0%)	130 (52%)	130 (52.0%)
None	26 (10.4%)	25 (10%)	32 (12.8%)
Occupation			
Currently unemployed	8 (3.2%)	8 (3.2%)	2 (0.8%)
Farming	91 (36.4%)	96 (38.4%)	99 (39.6%)
Service	21 (8.4%)	12 (4.8%)	16 (6.4%)
Business	37 (14.8%)	42 (16.8%)	47 (18.8%)
Self employed	57 (22.8%)	53 (21.2%)	42 (16.8%)
Other	10 (4.0%)	14 (5.6%)	12 (4.8%)
Marital Status			
Married	129 (51.6%)	147 (58.8%)	160 (64.0%)
Unmarried	114 (45.6%)	92 (36.8%)	89 (35.6%)
Others (Widow, Divorced, *etc.*)	7 (2.8%)	11 (4.4%)	1 (0.4%)
Education			
Illiterate	1 (0.4%)	0 (0.0%)	0 (0.0%)
Below high school	120 (48.0%)	121 (48.4%)	120 (48.0%)
High school	94 (37.6%)	96 (38.4%)	96 (38.4%)
Intermediate	22 (8.85%)	20 (8.0%)	21 (8.4%)
More than intermediate	13 (5.2%)	13 (5.2%)	13 (5.2%)

Table 2. Univariate comparison of somatometric measurements between three groups using Kruskal-Wallis test

Measurement	Chi-square	Asymptotic significance
MAHL	5.015	0.081
MAHB	15.263	< 0.001
MAHH	0.301	0.86
MFRB	1.451	0.484
BIZB	4.006	0.135
BIGB	23.859	< 0.001
MUFH	21.298	< 0.001
MTFH	11.471	0.003
PTFH	7.678	0.022
PUFH	28.17	< 0.001
NASL	16.228	< 0.001
NASB	1.014	0.602
NASD	11.801	0.003
MOUB	3.302	0.192
HELO	20.324	< 0.001
PEAL	6.852	0.033
PEAB	7.516	0.023
HVER	13.662	0.001
HACR	11.442	0.003
HILI	5.909	0.052
SHVE	17.146	< 0.001
BIAB	0.635	0.728
BICB	0.657	0.720
TCHB	2.848	0.241

Table 3. Pair-wise comparison of somatometric measurements between groups using Mann-Whiteny *U* test

Measurements	Pair of groups		
	Experimental and control-I	Experimental and control-II	Control-I and control-II
MAHB	0.0002	0.3655	0.0032
BIGB	0.0834	< 0.001	0.0017
MUFH	0.0024	< 0.001	0.1027
MTFH	0.0409	< 0.001	0.1937
PTFH	0.0066	0.0631	0.3501
PUFH	< 0.001	< 0.001	0.0336
NASL	< 0.001	< 0.001	0.8971
NASD	0.0216	0.3376	< 0.001
HELO	< 0.001	0.0001	0.71
PEAL	0.019	0.8111	0.0278
PEAB	0.0624	0.0079	0.4017
HVER	< 0.001	0.0524	0.0743
HACR	< 0.001	0.0209	0.2836
SHVE	0.0214	< 0.001	0.0874

Table 4. Correct classification (in %) obtained by robust LDA and robust QDA

Group	Model	
	LDA	QDA
Experimental	64.3	74.5
Control-I	66.0	69.6
Control-II	63.8	72.9
Overall	64.6	72.4

behavior has received very little attention either from anthropologists or from behavior scientists. Such studies involving criminals are too scanty indeed, presumably because crime, as such, not only is a multi-dimensional phenomenon but also carries socio-cultural bias in terms of the assigned cognizance of its severity. Hooton (*3*) reported a number of somatometric indices of criminals but mostly restricted the scope of somatometric measures to somatoscopic observations, while this study was mainly concerned with somatometric measurements of hard core as well as petty criminals and community people. Hooton (*3*) also reported excessive thinner body hair, thin beard, and broad ears in his sample of criminals as was found in the present study.

Among the somatometric dimensions included in the present study, significant differences between hard core criminals and community people were observed in respect to physiognomic ear breath. In addition to this characteristic among the studied samples of criminals, as compared to community people, other significant somatometric dimensions of hard core criminals, as found in the present study, were bigonial breadth, morphological upper facial height, morphological total facial height, physiognomic upper facial height, nasal length, height acromion, and sitting height vertex.

As compared to the petty criminals, significant somatometric dimensions of the hard core criminals, as found in the study were maximum head breadth, morphological upper facial height, morphological total facial height, physiognomic total facial height, physiognomic upper facial height, nasal length, nasal depth, physiognomic ear length, height vertex, height acromion, and sitting height vertex.

Also in the petty criminal group when compared to community people only maximum head breadth, bigonial breadth, physiognomic upper facial height, physiognomic ear length, and nasal depth were found significant.

The QDA model using the variables significant in the ANOVA provides the highest (74.5%) correct classification for the hard core criminal (experimental) group. For the petty criminal (control-I) group and community people (control-II) group the correct classification using the QDA model were 69.6% and 72.9%, respectively. Whereas when using the linear discriminant analysis model the correct classification obtained for the experimental, control-I, and control-II groups were 64.3%, 66.0%, and 63.8%, respectively. This blurred distinctiveness of these groups was recovered by using the discriminant analysis.

In conclusion, some of the somatometric measurements obtained were significantly different between the three groups and the blurred distinctiveness of these groups was captured through the quadratic discriminant analysis. These measurements along with psychological traits (such as behavior, environment, mental health of person, *etc*.) could be used to distinguish the criminals from the community people.

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Original Article

Unconjugated bilirubin modulates nitric oxide production *via* iNOS regulation

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Summary **To induce the *in vitro* endothelial dysfunction model, H5V cells were treated with tumor necrosis factor α (TNFα) and with unconjugated bilirubin (UCB) at two different physiological concentrations. The TNFα-induced reduction of nitric oxide (NO) concentration was reversed by UCB. Endothelial NO synthase (eNOS) gene expression was not influenced by treatments while inducible NO synthase (iNOS) expression was increased at 24 h. Co-treatment of H5V cells with pyrrolidine dithiocarbamate, TNFα (20 ng/mL) and UCB (Bf 15 or 30 nM) for 2 h caused a significant reduction of iNOS gene expression. We conclude that at physiological concentrations UCB prevents endothelial dysfunction by modulating NO concentration probably through inhibition of NF-κB.**

Keywords: Bilirubin, nitric oxide (NO), endothelial dysfunction

1. Introduction

Nitric oxide (NO) is one of the pivotal factors involved in the prevention of atherosclerotic lesions of the endothelium. It has been demonstrated that NO plays a critical role also in vascular hyper-permeability through both NO synthases (NOSs) expression, the endothelial form (eNOS) and an the inducible one (iNOS) *in vitro* (*1*) together with down-regulation of cytokine-induced endothelial cell adhesion molecule expression (*2*). Conversely, the inhibition of the NO producing enzyme eNOS causes an accelerated atherosclerotic process in experimental models (*3*).

Most probably eNOS has a double role in the pathogenesis of atherosclerosis. Under normal conditions, eNOS generates low concentrations of NO and probably peroxynitrite (*4*), favoring an anti-atherosclerotic environment (*5,6*). However, during hyperlipidemia and atherosclerosis, it may contribute to the formation of oxidative stress by a reduction in BH4-dependent NO formation and unopposed superoxide formation (*7*). In particular, in the setting of local induction, iNOS could favour the development of local toxic concentrations of peroxynitrite in atherosclerotic plaques (*8*).

Bilirubin, and in particular its unbound active form (unconjugated bilirubin; UCB), has been suggested to act as an endogenous tissue protector by attenuating radical-mediated damage to both lipids and proteins (*9*). There is increasing epidemiological evidence supporting an inverse association between cardiovascular disease and plasma levels of bilirubin (*10,11*). Recently we demonstrated that bilirubin may be protective in the development of atherosclerotic diseases by blunting the expression of E-selectin, vascular cell adhesion molecule (VCAM)-1 and intercellular adhesion molecule (ICAM)-1 (*12*) through a regulation of the NF-κB pathway (*13*). The earliest event that occurs in the development of atherosclerosis is characterized by a progressive modification in the physiological micro-environment identified as endothelial dysfunction (*14*). Endothelial dysfunction is a complex, multi-step mechanism where reduced NO levels have been reported as a marker (*5*) and, at the same time is characterized by increasing expression of adhesion molecules (AM).

Since the biological effects of UCB and the mechanisms of these effects on the development of atherosclerosis have yet to be explored, the aim of this study is to investigate the effect of UCB on NO production.

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2. Materials and Methods

2.1. *Materials*

Dulbecco's modified Eagles's medium high glucose (DMEM/HIGH), penicillin, and streptomycin were purchased from EuroClone, Siziano, Italy. Fetal calf serum was obtained from Invitrogen, Carlsbad, CA, USA. Chloroform, HPLC grade (99%), was obtained from Carlo Erba, Milan, Italy. Fatty acid free bovine serum albumin (BSA), dimethyl sulfoxide (DMSO, HPLC grade), UCB, tumor necrosis factor α (TNFα), and all other reagents and chemicals were purchased from Sigma-Aldrich, Milan, Italy.

2.2. *Cell cultures*

H5V cells, murine heart endothelial immortalized cells (*15*) (kind gift from Istituto Mario Negri, Milan, Italy), were grown to 80% confluence in DMEM/HIGH containing 10% (v/v) fetal calf serum, 100 U/mL penicillin, and 100 μg/mL streptomycin. When confluence was achieved, cells were washed three times with phosphate-buffered saline (PBS, pH 7.4) and incubated as described below.

2.3. *Studies of the cellular effects of UCB and cytokines*

UCB was purified as described by McDonagh and Assisi (*16*) and dissolved in DMSO (0.3 μL DMSO *per* μg UCB, and diluted with 21 mL serum free medium containing 30 μM BSA. Experiments were performed at unbound UCB concentrations (Bf) of 15 and 30 nM (*17*). To minimize photodegradation, all experiments with UCB were performed under dim lighting in vials wrapped in aluminium foil.

H5V cells were incubated in serum-free medium (DMEM/HIGH) containing BSA (30 μM) and DMSO (0.3%, v/v) with six different combinations of adducts: A) Control group, no adducts; B) TNFα group, add TNFα 20 ng/mL; C) UCB 15, add UCB to Bf of 15 nM; D) UCB 30, add UCB to Bf of 30 nM; E) co-treatment UCB15-TNFα, add UCB to Bf 15 nM and TNFα 20 ng/mL; and F) co-treatment UCB 30-TNFα, add UCB to Bf 30 nM and TNFα 20 ng/mL.

A further group of H5V cells were treated for 2 h with pyrrolidine dithiocarbamate (PDTC, 10 μM), a specific inhibitor of NF-κB (*18*), either alone or with UCB, as described above, in the presence or absence of TNFα (20 ng/mL) added 1 h after PDTC. PDTC was dissolved in serum free medium on the day of treatment, cells were then collected and mRNA extracted and real time RT-PCR performed as previously described (*12*).

2.4. *Mitochondrial toxicity determined by MTT test*

A stock solution of 3(4,5-dimethyltiazolyl-2)-2,5 diphenyl tetrazolium (MTT) was dissolved in PBS at 5 mg/mL (*19*). MTT solution was diluted to 0.5 mg/mL in DMEM/HIGH without phenol red. Cells were incubated with DMEM containing MTT for 2 h at 37°C at the end of the incubation period, the medium was replaced with 1 mL HCl containing 0.04 M isopropanol to dissolve MTT formazan crystals. Samples were then gently shaken for 2 h at 37°C. After centrifugation at 10,000 rpm for 3 min, absorbance at 570 nm was determined using a Beckman DU 640 spectrophotometer (Beckman Coulter S.p.A, Milan, Italy). Results were expressed as percentage of control cells, not exposed to UCB considered as 100% viability.

2.5. *LDH release test*

The presence of lactate dehydrogenase (LDH) in the culture supernatant and lysate cells was measured by a colorimetric assay, using the Cytotoxicity Detection Kit (LDH, Roche Applied Science, Penzberg, Germany) following the manufacturer's instructions. The absorbance of the samples at 490 nm was determined in a Beckman DU 640 spectrophotometer. Results were expressed as percentage of total LDH released *vs.* control cells, not exposed to UCB.

2.6. *Analysis of nitrite ion (NO^{2-}) levels*

Colorimetric assays with Griess reagent were used to detect NO levels. Culture media were saved and absorbance was determined in a spectrophotometer at 540 nm, values were compared against a standard curve with increasing concentrations of nitrite. Results were expressed as mean percentage values (%) of control cell group.

2.7. *RNA isolation and real-time RT-PCR analysis*

H5V monolayer cells were cultured on 6 well plates and pre-treated for 2 h with different UCB concentrations with or without TNFα (20 ng/mL). RT-PCR was performed as previously described (*12*). Briefly, total RNA was isolated using Tri Reagent solution (T9424, Sigma-Aldrich, Milan, Italy) and was quantified using a spectrophotometer at 260 nm. Retrotranscription was performed with an iScript cDNA Synthesis Kit (BioRad, Cat. No. 170-8891), the reaction was run in a thermocycler (Gene Amp PCR System 2400, Perkin-Elmer, Boston, MA, USA) at 25°C for 5 min, 42°C for 45 min, and 85°C for 5 min. Real-time PCR was performed according to the iQ SYBER Green Supermix (Bio-Rad) protocol. The selected host genes and their primer sequences (Table 1) were designed using Beacon Designer 2.0 software (PREMIER Biosoft International, Palo Alto, CA, USA). Reactions were run and analyzed on a Bio-Rad iCycler iQ Real-Time PCR detection system (iCycler IQ software, version 3.1; Bio-Rad).

Table 1. List of primer sequences

Murine Gene	Primer Foward	Primer Reverse
eNOS	GTGGAACAACTGGAGAAAGG	AAGGAGGCGAGGACTAGG
iNOS	TTGTGCGAAGTGTCAGTGG	TCCTTTGAGCCCTTTGTGC
β-Actin	CCTTCTTGGGTATGGAATCCTGTG	CAGCACTGTGTTGGCATAGAGG

Cycling parameters were determined, and resulting data were analyzed using the comparative Ct method as the means of relative quantitation, normalized to the housekeeping gene and expressed as $2^{-\Delta\Delta Ct}$. Melting curve analysis and gel electrophoresis was performed to assess product specificity.

2.8. *Statistical analysis*

All experiments were performed in triplicate and repeated at least three times. Results are expressed as means ± S.D. One way ANOVA with Tukey-Kramer post test was performed using GraphPad InStat version 3.00 for Windows 95 (GraphPad Software, San Diego, CA, USA). Probabilities < 0.05 were considered statistically significant.

3. Results and Discussion

3.1. *Effects of UCB on H5V cell viability*

The effect of UCB and TNFα on endothelial cell viability was evaluated by LDH release and mitochondrial toxicity determined by MTT assay. Plasma membrane integrity, investigated through LDH release, was unaffected by different doses of UCB (15 and 30 nM free bilirubin). As previously described (*20*), the addition of TNFα (20 ng/mL) significantly increased the extracellular LDH activity. However, no further effects were observed when co-treatment with TNFα and UCB were performed (Table 2).

On the contrary, UCB reduced, in a dose dependent manner, mitochondrial function (Figure 1) assessed by MTT assay indicating that the UCB effect was mainly due to a metabolic dysfunction but was not associated with increased cellular permeability or necrosis.

3.2. *NO concentration after treatment with TNFα alone or plus UCB*

We measured nitrite production after 24 and 48 h treatment with UCB and/or TNFα (20 ng/mL) (Figures 2A and 2B, respectively). The significant reductions of nitrite content in the cell supernatants induced by TNFα were not reversed by UCB after treatment for 24 h (Figure 2A) while UCB significantly reduced nitrite production after 48 h (Figure 2B) at both low and high concentrations (Bf 15 and 30 nM).

The regulation of NO metabolism and the enzymes involved in its synthesis (eNOS and iNOS) during the pro-inflammatory state is controversial (*6*). Cytokines are believed to induce the production of substantial amounts of NO by increasing iNOS expression and activity during the pro-inflammatory state (*21*). However, eNOS down-regulation by TNFα, and a decreased bio-availability of NO caused by endothelial dysfunction were also reported (*22*). Our data showed a reduction of NO levels by the pro-inflammatory cytokine TNFα after 24 and 48 h treatment (Figures 2A and 2B).

Table 2. Effects of UCB and TNFα on cell viability using LDH assay

UCB (Bf, nM)	LDH release (%)	
	– TNFα	+ TNFα
Control	14.9 ± 1.60	26.8 ± 1.30*
15	15.2 ± 1.30	23.1 ± 1.90*
30	14.6 ± 1.80	23.4 ± 1.70*

Results are expressed as % LDH released, $n = 3$ of one experiment out of two. * $p < 0.05$ *versus* – TNFα.

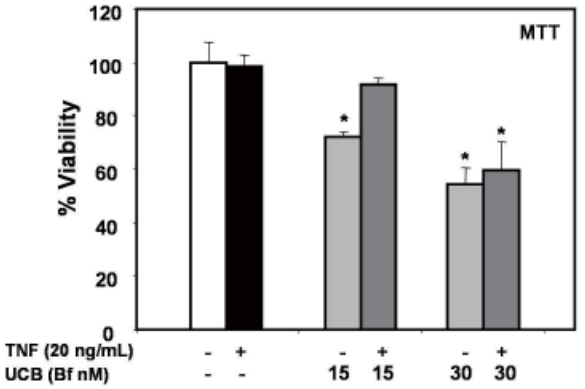

Figure 1. Effects of UCB on H5V cell viability by MTT assay. Results are expressed as mean percentage values (%) of three independent experiments performed in triplicate. Control cells (UCB, Bf 0 nM) were considered as 100%. * $p < 0.05$ *versus* controls.

3.3. *eNOS and iNOS expression after treatments with TNFα alone or plus UCB*

As shown in Figure 3, the gene expression of eNOS was not influenced by treatment with TNFα nor by different doses of UCB (15 and 30 nM) at 2, 6, and 24 h. As previously reported (*23*), TNFα increased the expression of iNOS at 2, 6, and 24 h, while no effect was observed with UCB treatment alone (data not shown). Co-treatments with TNFα and UCB indicated a slight but significant reduction of RNA expression of iNOS at 2 h while at 24 h they increased the gene expression of iNOS when compared with UCB or

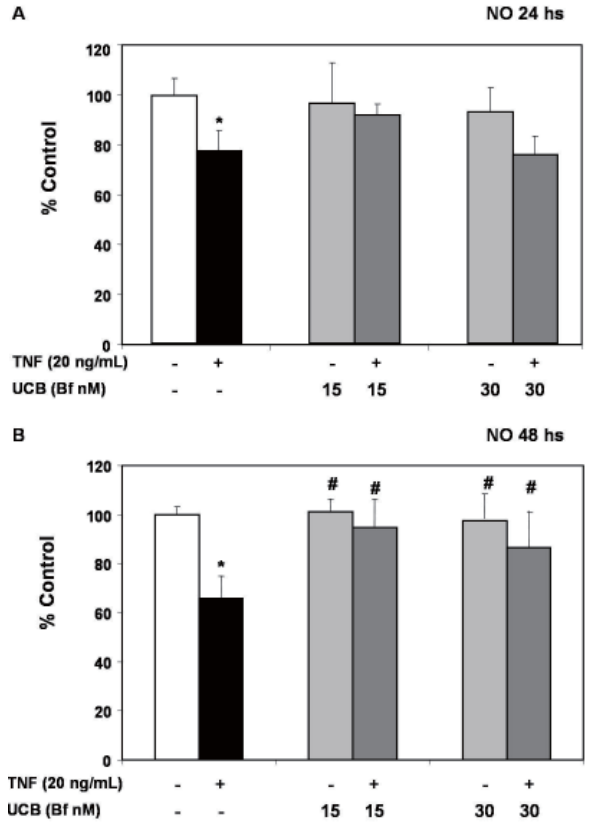

Figure 2. Effect of different doses UCB on NO production. Results are expressed as mean percentage values (%) of control cell group, from three independent experiments performed in triplicate. * $p < 0.05$ *versus* control; # $p < 0.05$ *versus* TNFα group alone (UCB, Bf 0 nM plus TNFα).

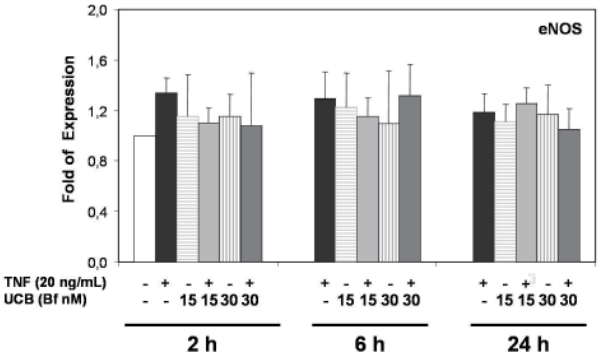

Figure 3. Effects of UCB on TNFα-induced gene expression of eNOS in H5V cells. Results are expressed as fold expression relative to respective untreated control set at 1.0.

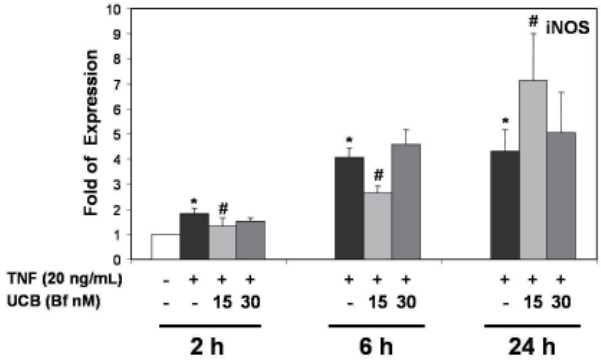

Figure 4. Effects of UCB on TNFα-induced gene expression of iNOS in H5V cells. Results are expressed as fold expression relative to respective untreated control set at 1.0. # $p < 0.05$ *versus* TNFα alone. * $p < 0.05$ *versus* controls.

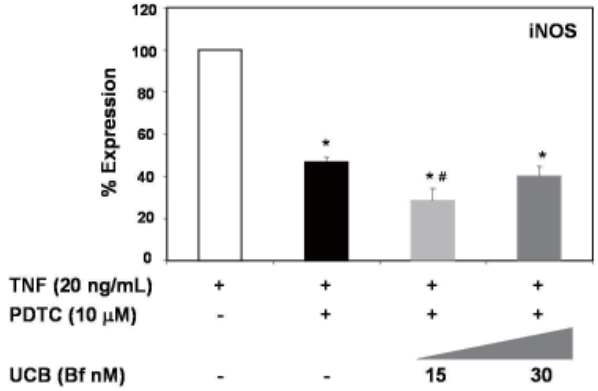

Figure 5. UCB and PDTC additively inhibit the overexpression of iNOS mRNA induced by TNFα. Results are expressed as percent expression, related to treatment with TNFα alone, considered as 100% (unshaded bar). * $p < 0.05$ *versus* treatment with TNFα alone; # $p < 0.05$ *versus* TNFα and PDTC treatments.

TNFα treatments alone (Figure 4).

Although the activity of UCB is basically related either to toxic or antioxidant effects according to its concentration (*24*), results obtained in the present and in our previous studies (*12,13*) demonstrate that UCB, even at physiological (15 nM) or mildly elevated (30 nM) concentrations, can modulate gene expression and endothelial cell function. Our data also demonstrate that UCB plays a role in the modulation of NO metabolism. The NO level is the result of a very complex regulated mechanism in which UCB may be involved at different steps. As shown in Figure 2, at 48 h, the NO level was reversed by UCB at both concentrations tested which suggests an up-regulation of iNOS induced by UCB at 24 h (Figure 4). However, UCB significantly inhibits iNOS expression at 2 h (Figure 4) when NO levels are not detectable (data not shown). This complex biphasic regulation of UCB with iNOS expression may be explained by a modulation of the NO levels. Since it was reported that NO levels are responsible for iNOS regulation itself (*25*), UCB may prevent NO induction when NO levels are not detectable. On the other hand, when NO levels are restored (at 48 h), UCB may compensate for these effects by a synergistic effect on iNOS induced TNFα (24 h). This hypothesis is further supported by the demonstration either *in vivo* and *in vitro*, that UCB limits the increase of hepatic levels of TNFα, NO, and iNOS caused by treatment with endotoxin (*26*).

Several signaling pathways, in particular NF-κB, are described as involved in regulating the gene expression of iNOS and adhesion molecules (*27*). As previously reported (*13*), UCB does not affect NF-κB translocation but inhibits the nuclear translocation of NF-κB induced

by TNFα. We found that PDTC, an IkBα inhibitor that prevents release of p65 NF-κB (*28*), has an additive effect with UCB on the inhibition of TNFα-induction of iNOS after 2 h of treatment (Figure 5).

The present results further reinforce the putative role of bilirubin in the prevention of tissue injury in response to inflammatory stimuli and, in agreement with our recent studies (*12,13*) and others on heme oxygenase-1 (*29*), indicate UCB is a potential cardiovascular protective factor.

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Original Article

Genetic diversity of the *Helicobacter pylori* sialic acid-binding adhesin (*sabA*) gene

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Summary **A putative virulence factor, SabA, a sialic acid-binding adhesin, has recently been characterized in *Helicobacter pylori* from European isolates. However, little genetic information is available for *sabA* genes in strains isolated from Japanese patients. Here, we investigated the presence of the *sabA* gene in 23 *H. pylori* clinical isolates using polymerase chain reaction detection. It was found that 91.3% of *H. pylori* isolates examined contain the *sabA* gene. Sequence comparison and phylogenetic analysis based on the deduced amino acid sequence of *sabA* in nine *H. pylori* isolates from Japanese patients and three *H. pylori* strains from Western individuals suggested that *sabA* is genetically diverse and the clustering of the strains based on SabA is related to their geographical origin. It needs to be further assessed whether the genetic diversity of *sabA* is associated with the clinical outcomes of *H. pylori* infection.**

Keywords: *Helicobacter pylori*, SabA, PCR, phylogenetic tree

1. Introduction

Helicobacter pylori (*H. pylori*) is a human gastroduodenal pathogen that affects at least half the world's population. Colonization of this bacterium in the stomach mucosa results in chronic mucosal inflammation without any clinical symptoms in the majority of infected people. In approximately 10% of infected individuals, however, the chronic inflammation causes a diverse spectrum of gastric diseases, ranging from peptic ulcer to gastric cancer and mucosa-associated lymphoid-tissue (MALT) lymphoma as well (*1*,*2*). Although it is yet unclear what exactly determines the clinical outcomes of *H. pylori* infection, a complex interaction of bacterial virulence factors, host immune response and environmental influences is thought to contribute to different clinical outcomes in *H. pylori* infection (*3*,*4*).

Among the bacterial virulence factors identified so far, *H. pylori* cytotoxin-associated gene A (*CagA*), its related pathogenicity island (*CagPAI*), and vacuolating cytotoxin A (*VacA*) are the best characterized toxins that are strongly linked to the pathogenicity of *H. pylori* (*5*). We currently know that CagA is delivered into gastric epithelial cells by a type IV bacterial secretion system (*6*,*7*). Within gastric epithelial cells, CagA is usually phosphorylated by host cell kinases, and it interacts with multiple host signaling molecules, which result in the morphological changes. Nonphosphorylated CagA can interact with certain host cell proteins and elicit numerous cellular effects (*8-10*), including disruption of tight cell-to-cell junctions, loss of cell polarity, and pro-inflammatory responses. Unlike CagA, VacA is secreted by *H. pylori* and it induces massive vacuolization in gastric epithelial cells (*5*). Recent studies demonstrated that VacA not only causes functional alterations such as increased membrane permeability and apoptosis in gastric epithelial cells but also has multiple effects on both T and B cells (*11*,*12*).

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In addition to these virulence factors, *H. pylori* adhesins such as the Lewis blood group antigen-binding adhesin (BabA) (*13*) and the sialic acid-binding adhesin (SabA) (*14*) are widely believed to play a critical role in initial colonization of *H. pylori* and subsequent persistence of infection. Over the past decade, extensive studies on adherence properties of *H. pylori* have demonstrated that BabA mediates strong binding of the bacteria to gastric epithelial cells and facilitates efficient delivery of virulence factors such as CagA and VacA into host cells (*5,15*). Another adhesin, SabA, which was identified and purified from the *babA*-mutant *H. pylori* strain by Mahdavi *et al*. (*14*), was shown to mediate a weaker binding of the bacteria to sialylated glycoconjugates expressed in inflamed gastric epithelium. Recently, it has also been found that SabA binds specifically to sialylated carbohydrates on the surface of neutrophils and thereby induces pro-inflammatory and immune responses (*5,16*), suggesting that SabA might play an important role in determining the clinical outcome of *H. pylori* infection.

Indeed, epidemiological studies from European and North American countries have revealed the close relationship between gastric cancer and SabA positive status (*14,17-19*). Two reports from Asia (*20,21*), however, indicated that SabA status has little influence on the clinical outcome of *H. pylori* infection. It is not yet known whether the inconsistent result is due to geographic or disease-associated allelic variation in *H. pylori* because there is little genetic information available for *sabA* genes from strains around the world. In the present study, we investigated the presence of the *sabA* gene in *H. pylori* isolates from Japanese patients. Moreover, the nucleotide sequences of the *sabA* gene in ten *H. pylori* isolates from our patients were determined and compared with those of the three *H. pylori* strains whose genome sequences were released previously in the GenBank database (*22-24*).

2. Methods

2.1. *H. pylori clinical isolates*

All *H. pylori* strains used in the study were deep-frozen primary pure *H. pylori* strains isolated from 23 outpatients with gastroduodenal ulcers who underwent upper endoscopy in our university hospital between 2001 and 2003.

2.2. *Culture of H. pylori*

An aliquot of the frozen primary pure *H. pylori* strains described above was thawed and inoculated onto the Pourmedia Vi HELICO AGAR plates (Eiken, Tokyo, Japan). Plates were incubated in a microaerophilic atmosphere with 5-8% CO_2 at 37°C for up to 5 days. The colony was picked up by a toothpick, and transported to a vial containing 20 μL of 1× phosphate-buffered saline (PBS, pH 7.0) and stored at –80°C until polymerase chain reaction (PCR) was performed.

2.3. *Detection of sabA gene by PCR*

The presence of *sabA* in *H. pylori* clinical isolates was analyzed by PCR using three primer pairs separately. Primer pairs 1, 3Fm (5'-CCGCTAGTGTCCAGGGTAAC-3')-5Rm (5'-CACCGCGTATTGCGTTGGGTA-3'), pair 2, 1Fm (5'-GGCTCTAGCAATGTGTGGCAG-3')-5Rm, and pair 3, 3Fm-1Rm (5'-CGCGCTGTAAGG GTTATTGAAC-3'), were used to amplified different fragments of *sabA* gene as reported by Mahdavi *et al*. with mild modification (*14*). Briefly, each amplification was performed in a reaction volume of 25 μL with GeneAmp 10× PCR Buffer (Applied Biosystems, Carlsbad, CA, USA), 1 unit of AmpliTaq Gold DNA Polymerase (Applied Biosystems), 0.2 μM of dNTPs, with 0.2 μM of the forward and reverse primers and with 5 μL *H. pylori* solution from each sample. The amplification consisted of an initial denaturation step at 95°C for 10 min, followed by 35 cycles, including a denaturation step at 94°C for 20 sec, annealing at 45°C for 20 sec, and extension at 72°C for 90 sec. The final extension was performed for 5 min at 72°C. The resulting PCR product was analyzed on 1% agarose gel stained with ethidium bromide.

2.4. *DNA sequence analysis*

The amplified DNA was purified with a QIA quick PCR purification kit (QIAGEN, Hilden, Germany), and directly sequenced using an ABI Prism Bigdye terminator v1.1 cycle sequencing kit (Applied Biosystems). Sequence analysis was performed utilizing programs of the GENETYX package (version 9.0; Genetyx Co., Tokyo, Japan). The evolutionary relationship among different *H. pylori* isolates was elucidated by the 6-parameter method. The phylogenetic tree was constructed with the neighbor-joining method in the ODEN program (version 1.1) (*25*).

3. Results

The presence of the *sabA* gene in 23 *H. pylori* clinical isolates was detected by PCR. We utilized the same primers as described by Mahdavi *et al*. with three different combinations for PCR detection (Figure 1) because there is very little sequence data available for the *sabA* gene in Japanese isolates. The PCR amplification with primer pairs 1, 2, and 3 yielded products of 364, 1,087, and 1,330 bp in length, respectively. As shown in Table 1, three PCR assays yielded different positive rates for the *sabA* gene in 23 *H. pylori* isolates, with the highest one (91.3%) using primer pair 1, suggesting that there is considerable sequence diversity of the *sabA* gene

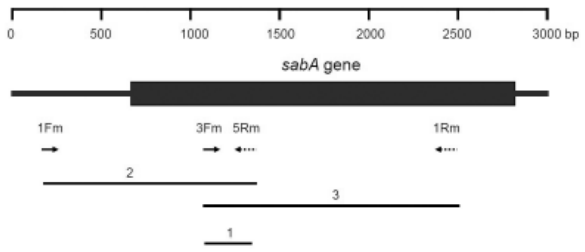

Figure 1. Detection of *sabA* gene by PCR. The line at the top indicates the gene length. The black rectangle under the ruler indicates the *sabA* gene. The numbered solid lines below represent the fragments that were amplified by different primer pairs. Solid and dashed arrows represent foreword and reverse primers used in this study.

Table 1. Detection of *sabA* gene by PCR in 23 *H. pylori* clinical isolates

Number of *H. pylori* strains	PCR results (n = 23)		
	Primer pair 1	Primer pair 2	Primer pair 3
Y03	+	+	+
Y04	+	+	–
Y06	+	–	+
Y07	+	+	–
Y09	–	+	–
Y11	+	–	–
Y13	+	+	–
Y14	+	+	+
Y15	+	+	+
Y16	–	–	–
Y17	+	+	–
Y19	+	+	+
Y21	+	–	–
Y22	+	+	+
Yno1	+	+	+
Y26	+	–	–
Yno3	+	+	+
Y28	+	–	–
Y29	+	+	–
Y31	+	+	–
Y37	+	+	+
Y38	+	+	+
Y40	+	+	+
Number of *sabA*-genopositive	21	17	11

in *H. pylori* clinical isolates.

In order to evaluate the genetic diversity of *sabA*, the fragments amplified by PCR with primer pairs 2 and 3 were subjected to sequence analysis in ten *H. pylori* isolates. Nucleotide point mutations, deletions, and insertions were commonly observed in the *sabA* gene. Tables 2 and 3 summarize the homology of *sabA* nucleotide and amino acid sequences among different *H. pylori* strains. The amino acid sequence diversity of SabA ranged from 5.1% to 13.3% in the *H. pylori* clinical isolates analyzed.

Figure 2 shows a phylogenetic tree constructed using the amino acid sequences of SabA in nine *H. pylori* isolates from Japanese patients and three *H. pylori* strains from European and American patients whose genomic sequences were released in the GenBank database. The pattern clearly indicated that the *H. pylori* isolates in Japan are more closely related to each other than to those from Western countries, suggesting that *sabA* genes may be divided into two groups, a Western group and a Japanese group.

4. Discussion

H. pylori virulence factors, such as urease, CagA, VacA, or BabA, might account for the development of different gastric diseases, including gastritis, peptic ulcer, and gastric cancer (*1,11*). Recent studies from Europe provided evidence that another adherence factor, SabA, may contribute further to the enhanced pathogenicity of *H. pylori* in human gastric epithelium (*14*). However, previous studies on *H. pylori* have revealed that clinical relevance of virulence factors observed in Western individuals vary apparently from those in Asian populations, due in large part to the remarkable genetic diversity of *H. pylori* (*26*). Therefore, the presence and characteristics of the *sabA* gene in *H. pylori* clinical isolates in Japan needed to be assessed.

In the present study, we used three primer sets whose sequences were derived from European strains to detect the *sabA* gene, using PCR, in the *H. pylori*

Table 2. Comparison of nucleotide sequence homology of *sabA* genes (%)

Strain No.	Y14	Y5	Y19	Y22	Y37	Y38	Y40	Yno1	Yno3	26695	J99	HPAG1
Y03	93.7	94.5	92.8	93.7	93.5	93.6	93.2	94.5	93.2	89.2	89.4	89.5
Y14		94.4	94.0	94.5	94.3	95.3	94.1	93.8	95.0	88.7	90.3	89.4
Y15			92.8	94.5	93.9	95.8	94.2	92.0	92.9	88.5	89.8	89.0
Y19				94.0	93.9	93.5	93.8	92.5	93.9	89.3	90.0	90.3
Y22					94.8	94.8	94.9	92.9	94.0	89.2	89.9	89.4
Y37						95.0	95.0	93.4	94.8	88.6	89.3	88.8
Y38							93.9	93.0	93.7	88.9	90.0	89.4
Y40								92.4	93.7	89.2	89.8	90.0
Yno1									93.3	89.6	89.4	89.7
Yno3										88.7	90.0	88.7
26695											91.9	94.3
J99												91.9

The *H. pylori sabA* gene sequences determined in this study have been submitted into GenBank with accession number AB244057-AB244066.

Table 3. Comparison of amino acid sequence homology of SabA (%)

Strain No.	Y14	Y15	Y19	Y22	Y37	Y40	Yno1	Yno3	26695	J99	HPAG1
Y03	91.6	91.7	90.9	90.9	91.1	90.9	92.5	91.3	87.2	88.3	87.3
Y14		92.5	93.2	93.2	92.6	92.9	92.0	93.2	87.6	88.6	87.8
Y15			92.0	94.3	91.7	92.8	90.1	90.9	87.3	89.0	88.7
Y19				93.2	93.3	93.6	90.7	92.4	88.2	88.4	88.3
Y22					93.2	93.9	90.5	91.8	88.6	88.9	89.0
Y37						93.3	90.8	92.7	87.4	88.0	87.5
Y40							90.2	92.3	87.7	88.6	88.7
Yno1								90.8	88.7	88.8	88.2
Yno3									86.7	89.0	86.9
26695										90.9	94.9
J99											90.6

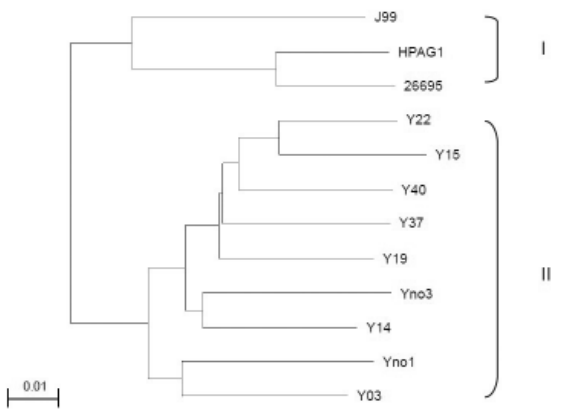

Figure 2. A phylogenetic tree constructed using the amino acid sequences of *H. pylori* SabA in 9 clinical isolates from Japanese patients and 3 strains from the GenBank database. The length of the horizontal bar indicates the number of amino acid substitutions per site.

strains isolated from Japanese patients. As shown in Table 1, the *sabA* gene was detected in 21 of 23 isolates using PCR with primer pair 1, while 17 and 11 out of 23 isolates with primer pair 2 and pair 3, respectively. These data suggested that 1) most, if not all, *H. pylori* isolates in the current study contain the *sabA* gene; and 2) the *sabA* gene is diverse, which causes ambiguous PCR results. Hence, it is necessary to design new primers based on the conserved sequences of *sabA* in Japanese *H. pylori* isolates for further epidemiological studies.

Sequence analysis showed considerable sequence diversity of the *sabA* gene among *H. pylori* clinical isolates, ranging from 5.0% to 11.5% at the nucleotide level and 5.1% to 13.3% at the amino acid level. Phylogenetic analysis demonstrated clustering of strains according to their geographical origin; cluster I contained all isolates from Japan whereas cluster II consisted of three isolates from Western countries. From these observations, two questions arose: 1) whether SabA is associated with pathogenicity of *H. pylori* given that *sabA*-genopositive strains were prevalent in Japanese patients studied here, and 2) what is the clinical relevance of the genetic diversity in *sabA*. Recently, Yanai *et al*. reported a high prevalence of functional *sabA* (81%) in 108 Japanese clinical *H. pylori* isolates based on sequence analysis of the CT dinucleotide repeats numbers at the 5' region of *sabA* gene, and indicated a close relationship between *sabA* status and atrophic gastritis. Nevertheless, there is increasing evidence that complicated regulatory mechanisms are involved in control of expression of the *sabA* gene. A report from Taiwan based on Western blotting analysis on 145 *H. pylori* clinical isolates indicated that only 31% of the isolates expressed SabA. Hence, to assess the clinical relevance of the *sabA* gene diversity, further genetic epidemiology studies on SabA and other virulence factors are needed.

Acknowledgements

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Original Article

Inhibition of *C35* gene expression by small interfering RNA induces apoptosis of breast cancer cells

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Summary **C35 was reported to be a new biomarker and therapeutic target for breast cancer. To explore the functional importance of C35, we constructed small interfering RNA (siRNA) targeting *C35* and investigated the effects of the siRNAs on C35 expression and apoptosis of T47D cells. *C35* siRNAs were constructed and named psiRNA-*C35*-1 and psiRNA-*C35*-2. Reverse transcription-polymerase chain reaction (RT-PCR) and Western blots were used to detect the effects of the siRNAs on mRNA and protein expression of C35 in T47D cells. The effects of the two siRNAs on apoptosis of T47D cells were detected by flow cytometry and terminal dUTP nicked-end labelling assays. Also, the apoptosis related molecule caspase-3 was detected using Western blots. The psiRNA-*C35*-1 and psiRNA-*C35*-2 siRNAs were verified by both *Eco*R I/*Hin*d III digestion analysis and automated DNA sequencing. RT-PCR and Western blots showed that *C35* mRNA and protein expression in T47D cells were obviously inhibited after psiRNA-*C35*-1 and psiRNA-*C35*-2 transfection. Flow cytometry and terminal dUTP nicked-end labelling assays showed that apoptosis of T47D cells was significantly induced after transfection with psiRNA-*C35*-1 and psiRNA-*C35*-2 ($p < 0.05$). Also, caspase-3 expression in the psiRNA-*C35*-1 and psiRNA-*C35*-2 transfected cells was obviously higher than that of the Lipofectamine and pTZU6+1 transfected cells. This study showed that apoptosis of T47D cells can be significantly induced by inhibiting C35 expression using siRNAs, which may be caused by activating caspase-3. C35 might play an important role in apoptosis of breast cancer cells, and therapeutic strategies targeting *C35* may be useful for breast cancer treatment.**

Keywords: C35, apoptosis, small interfering RNA (siRNA), breast cancer

1. Introduction

Breast cancer is the most common cancer among women except for non-melanoma skin cancer and is the second leading cause of cancer-related deaths in women today (*1*). Although early diagnosis approaches and proper management, including various options of evidence-based treatment have not only reduced mortality but also enhanced patients' quality of life, the mortality rate due to breast cancer in the world has continued to increase, and the number of patients is also increasing rapidly (*2*). Identification of phenotypic and genetic changes that are conserved throughout breast cancer disease progression may help to illuminate key events necessary to transform healthy cells, maintain malignancy, and facilitate development of metastases (*3*).

C35, a newly discovered gene, is located on chromosome 17q12 adjacent to the oncogene that encodes human epidermal growth factor receptor 2 (HER2/neu) (*4*). The *C35* gene encodes a small 115 amino acid protein with a molecular weight of ~ 12 kDa that has no sequence similarity with any known genes or proteins, and its functional importance is presently unknown (*5,6*). Immunohistochemical studies in breast cancer lumpectomy samples showed that the *C35* gene was over-expressed in more than 60% of breast cancer tissues, and was not evident in any normal tissues in

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women (*5*). They also confirmed the overexpression of C35 in *HER2/neu*-negative breast cancer patients and breast tumor cell lines, suggesting independent transcriptional control mechanisms for C35 (*5*). These studies suggested that C35 protein might be an important indicator of breast cancer. However, to establish C35 as a biomarker and therapeutic target for breast cancer treatment, it is of the utmost importance to explore the function of this novel protein (*7*).

RNA interference (RNAi) is a potent gene silencing mechanism conserved in most eukaryotic organisms for RNA-guided regulation of gene expression, in which double stranded ribonucleic acid inhibits the expression of specific genes with complementary nucleotide sequences (*8*). RNAi has been applied for functional genomic studies in a variety of areas, including cancer research, by facilitating a better understanding of the mechanisms that underlie tumorigenicity and the identification of novel factors that either promote or inhibit oncogenic transformation (*9*). RNAi mediated by small interfering RNAs (siRNAs) is a powerful technology allowing the silencing of mammalian genes with great specificity and potency (*10,11*). In this study, to explore the functional importance of C35, we constructed siRNAs targeting *C35* and investigated the effects of siRNA constructs on C35 expression, as well as apoptosis of T47D cells.

2. Materials and Methods

2.1. *Materials*

Plasmid pTZU6+1 was received as a gift from Dr. David Engelke, University of Michigan, Ann Arbor, MI, USA. RNA oligonucleotides were synthesized by Biological Engineering Co., Shanghai, China. Human breast cancer cell line T47D, was obtained from Institute of Basic Medicine, Shandong Academy of Medical Sciences, Ji'nan, China. Annexin V-FITC Apoptosis Detection Kit was purchased from BD Biosciences, San Jose, CA, USA. TdT-mediated dUTP-biotin nick end labeling (TUNEL) Apoptosis Detection Kit was purchased from Roche, Madison, WI, USA. Rabbit anti-human C35 monoclonal antibody was purchased from Invitrogen, Grand Island, NY, USA.

2.2. *Cells and cell culture conditions*

T47D cells were incubated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% of fetal bovine serum (FBS), 100 U/mL of penicillin, and 100 U/mL of streptomycin in a humidified atmosphere with 5% CO_2 at 37°C. For maintenance and subculture, T47D cells in exponential phase were treated with 0.25% trypsin solution containing 0.02% EDTA. After reaching 80% confluence, the cells were collected.

2.3. *siRNAs*

Two pairs of oligonucleotides targeting two different regions of *C35* were designed according to *C35* sequence in GenBank (NM_032339) following the rules of Tuschl T (*12*). The two regions within the *C35* gene were as follows: siRNA-1 (position 259-278), sense: 5'-TCTGAAGATCTCATTGAGGCCATCTTCG GATGGCCTCAATGAGATCTTTTT-3'; antisense: 5'-CTAGAAAAAAGATCTCATTGAGGCCATCCGAA GATGGCCTCAATGAGATCTC-3'; siRNA-2 (position 282-300), sense: 5'-TCGAGGAGCCAGTAATGGAG AAACTTCGGTTTCTCCATTACTGGCTCTTTTT-3'; antisense: 5'-CTAGAAAAAGAGCCAGTAATGGAG AAACCGAAGTTTCTCCATTACTGGCTCC-3'. The underlined parts were the *Xba* I and *Xho* I degestion sites and the stem-loop structures. The four oligodeoxyr ibonucleotides encoding two siRNAs were dissolved in annealing buffer, 95°C for 5 min, and cooled gradually to room temperature to anneal.

2.4. *Construction*

siRNA expression vectors were prepared using the pTZU6+1 vector. The pTZU6+1 was digested with *Sal* I and *Xba* I and then ligated with the annealed oligodeoxyribonucleotides, yielding the pTZU6+1-*C35* constructs, named, psiRNA-*C35*-1 and psiRNA-*C35*-2. Ligation mixtures were then transformed into *E. coli* DH5α. Ampicillin-resistant clones were picked and expanded. The recombinant plasmids were extracted according to the manufacturer's protocol of QIAGEN EndoFree plasmid Maxi Kit (Qiagen, Hilden, Germany). After double digestion with *Eco*R I and *Hin*d III, the fragments were verified using 1% agarose gel analysis. The constructed plasmids were also confirmed by DNA sequencing (Biological Engineering Co., Shanghai, China).

2.5. *In vitro transfection*

On the day before transfection, cells were collected and reseeded in 6-well plates at a density of 4 × 10^5/mL in serum-free DMEM medium. After 24 h, the cells were transfected using Lipofectamine™ 2000 according to previous studies (*11*). In brief, the cells were incubated with a mixture of 4 μg of DNA (pTZU6+1 control, psiRNA-*C35*-1, or psiRNA-*C35*-1) and diluted with Lipofectamine 2000 reagent, or only Lipofectamine 2000 for 4 h at 37°C. Transfection medium was then replaced with DMEM medium including 10% FBS and incubated for 48 h at 37°C. The transfection rates were measured using fluorescence microscopy.

2.6. *Determination of C35 mRNA expression levels by reverse transcription-polymerase chain reaction (RT-PCR)*

Semi-quantitative RT-PCR was used to detect the effects of psiRNA-*C35*-1 and psiRNA-*C35*-2 on the expression of *C35* mRNA (*13*). Briefly, 72 h after transfection, total RNA was isolated using TRIZOL reagents according to the manufacturer's protocol. Isolated total RNA was first reverse transcribed into cDNA using random primers and SuperScript™ II reverse transcriptase. Then cDNA was used as templates for amplification in PCR. Sequences of primers were as follows: for *C35*, sense, 5'-CGGAATTCATGAGCGGG GAGCCGG-3'; antisense, 5'-CGGGATCCTCACAGG ATGACGCGGGAGGA-3'; for *β-actin*, sense, 5'-GTGG GGCGCCCCAGGCACCA-3'; antisense, 5'-CTCCTTA ATGTCACGCACGATTTC-3'. PCR cycling parameters were: at 95°C for 5 min to pre-denaturation, followed by 35 cycles at 94°C for 1 min, 60°C for 30 sec, and 72°C for 1 min. *β-actin* was used as an internal control. The PCR products were analyzed by electrophoresis on a 1.2% ethidium bromide (EB)-agarose gel and viewed under UV. The ImageMaster TotalLab software (GE Healthcare Bio-Sciences, Piscataway, NJ, USA) was used to measure the intensity of each band.

2.7. *Determination of C35 protein expression levels by Western blots*

For Western blots, 72 h after transfection, cells were lysed with RIPA buffer for 30 min at 4°C, and debris was removed by centrifugation at 20,000 × g for 10 min. Equal amounts of protein (20 μg) in the cell extracts were fractionated on 10% sodium dodecyl sulfate polyacrylamide gels followed by transfer to polyvinylidene difluoride membranes. Membranes were incubated for 1 h in 5% skim milk in PBS with monoclonal antibodies (C35: 1:1,000, capase-3: 1:1,000, or β-actin: 1:2,000) followed by incubation with secondary antibodies (1:4,000) for 1 h. Proteins were visualized with enhanced chemiluminescence using the ECL Western Blotting Starter Kit (GE Healthcare Bio-Sciences, Piscataway, NJ, USA).

2.8. *Apoptosis assays*

Apoptosis was quantified by detecting surface exposure of phosphatidylserine in cells using an Annexin V-FITC kit (*14*). Briefly, after transfection for 72 h, cells were stained with 3 mL of Annexin V and 1 mL of propidium iodide (PI; 1 mg/mL) in 100 mL of Annexin V binding buffer (10 mM HEPES, pH 7.4, containing 140 mM NaCl and 5 mM $CaCl_2$) and incubated for 15 min at room temperature in the dark. Subsequently, 400 mL of binding buffer was added and mixed gently. Cells were then observed using flow cytometry.

TUNEL assay was used to observe the cell morphology in culture. Briefly, cells (fixed in 4% paraformaldehyde for 20 min) were blocked with 3% H_2O_2 in methanol for 10 min at room temperature and permeabilized in ice-cold 0.1% Triton X-100, 0.1% sodium citrate for 2 min. TUNEL reaction mix was added and incubated for 1 h at 37°C in the dark under humidified conditions. TUNEL-Peroxidase (POD) converter was added and incubated for an additional 30 min in the dark under humidified conditions. Cells were then visualized with a Nikon Eclipse E600 microscope and photographed with a Photometrics Cool Snap ES camera.

3. Results

3.1. *Construction of psiRNA-C35*

The recombinant plasmids, named psiRNA-*C35*-1 and psiRNA-*C35*-2, were purified from transformed *E. coli*, and verified by *Eco*R I/*Hin*d III digestion analysis and automated DNA sequencing. Recombinant psiRNA-*C35* vectors which contain the siRNA coding sequences should yield new 400-bp fragments after double digestion with *Eco*R I/*Hin*d III. This was verified by agarose gel analysis. As shown in Figure 1, the positive psiRNA-*C35* constructs can yield new 400-bp fragments, while the negative control vectors can only yield 350-bp fragments. The inserted sequences in psiRNA-*C35*-1 and psiRNA-*C35*-2 were verified by automated DNA sequencing. The sequences were: psiRNA-*C35*-1, 5'-CGAAACACCGTCGAGAG ATCTCATTGAGGCCATCTTCGGATGGCCTCAAT GAGATCTTTTTTCTAGAGCGGACTTCGGTCCGC TTTTTACTAGGACCTGCAGGCATGCAAGCTTGG CACTGGCCGTCGTTTTACAACGTCGTGACTGG GAAAAC-3'; psiRNA-*C35*-2, 5'-TCTTGGCTTTATAT ATCTTGTGGAAAGGACGAAACACCGTCGAGGA GCCAGTAATGGAGAAACTTCGGTTTCTCCATTA CTGGCTCTTTTTCTAGAGCGGACTTCGGTCCGC TTTTTACTAGGACCTGCAGGCATGCAAGCTTGG

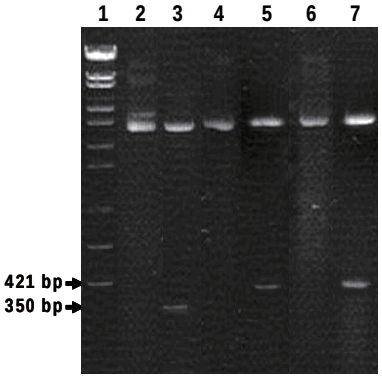

Figure 1. Restriction enzyme digestion analysis of recombinant plasmids psiRNA-*C35*-1, psiRNA-*C35*-2, and pTZU6+1 control. 1, Marker; **2**, pTZU6+1; **3**, pTZU6+1 with *Eco*R I and *Hin*d III; **4**, psiRNA-*C35*-1; **5**, psiRNA-*C35*-1 with *Eco*R I and *Hin*d III; **6**, psiRNA-*C35*-2; **7**, psiRNA-*C35*-2 with *Eco*R I and *Hin*d III.

CACTGGCCGTCGTTTTACAACGTCGTGACTGG GAAAACCCTGGCGTTACCCAACTTAATC-3'. The underlined parts are where the DNA sequences were inserted. These results showed that both psiRNA-*C35*-1 and psiRNA-*C35*-2 were successfully constructed.

3.2. *Effects of psiRNA-C35 constructs on C35 mRNA expression in T47D cells*

The effects of psiRNA-*C35*-1 and psiRNA-*C35*-2 on the expression of *C35* mRNA were detected by semi-quantitative RT-PCR. As shown in Figure 2, the bands of pTZU6+1 and Lipofectamine-transfected cells were much more obvious than that of the psiRNA-*C35*-1 and psiRNA-*C35*-2-trasfected cells. Normalized *C35* mRNA levels of T47D cells transfected with pTZU6+1, psiRNA-*C35*-1, and psiRNA-*C35*-2 were 95.3%, 40.0%, and 28.4%, respectively, as compared with that of T47D cells transfected with Lipofectamine (100.0%). This result suggested that expressed siRNAs psiRNA-*C35*-1 and psiRNA-*C35*-2 could effectively inhibit *C35* mRNA expression. Additionally, the effect of psiRNA-*C35*-2 on *C35* mRNA expression was more obvious than that of psiRNA-*C35*-1.

3.3. *Effects of psiRNA-C35 constructs on C35 protein expression in T47D cells*

Effects of psiRNA-*C35*-1 and psiRNA-*C35*-2 on expression of C35 protein were detected by Western blots. As shown in Figure 3, the bands of pTZU6+1 and Lipofectamine-transfected cells were much more obvious than that of the psiRNA-*C35*-1 and psiRNA-*C35*-2-transfected cells. Normalized C35 protein levels of T47D cells transfected with pTZU6+1, psiRNA-*C35*-1, and psiRNA-*C35*-2 were 94.9%, 29.6%, and 32.2%, respectively, as compared with that of T47D cells transfected with Lipofectamine (100.0%). This result suggested that siRNAs expressed by psiRNA-*C35*-1 and psiRNA-*C35*-2 could effectively inhibit C35 protein expression. Additionally, the effect of psiRNA-*C35*-2 on C35 protein expression was more obvious than that of psiRNA-*C35*-1.

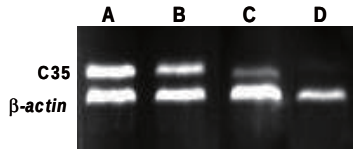

Figure 2. Semi-quantitative RT-PCR of *C35* and *β-actin* expression in T47D cells infected with a Lipofectamine control, pTZU6+1, psiRNA-*C35*-1, and psiRNA-*C35*-2. A, Lipofectamine control; **B**, pTZU6+1; **C**, psiRNA-*C35*-1; **D**, psiRNA-*C35*-2.

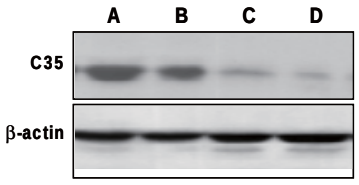

Figure 3. Western blot analysis of C35 expression in T47D cells infected with a Lipofectamine control, pTZU6+1, psiRNA-*C35*-1, and psiRNA-*C35*-2. A, Lipofectamine control; **B**, pTZU6+1; **C**, psiRNA-*C35*-1; **D**, psiRNA-*C35*-2.

3.4. *Apoptosis assays*

To evaluate the impact of silenced *C35* expression on apoptosis of T47D cells, apoptosis rates were detected using an Annexin V-FITC kit. The result showed that the apoptosis rates of the Lipofectamine, pTZU6+1, psiRNA-*C35*-1, and psiRNA-*C35*-2 transfected cells were 18.5%, 21.6%, 33.7%, and 44.9%, respectively. The apoptosis rates of the psiRNA-*C35*-1 and psiRNA-*C35*-2 transfected cells were significantly higher than that of the Lipofectamine and pTZU6+1 transfected cells ($p < 0.05$) (Figure 4). The apoptosis of T47D cells might be induced by the decreased expression of *C35* mRNA and protein.

Cell morphology in culture was observed using a TUNEL assay. In the Lipofectamine and pTZU6+1 transfected cells, most of the cells with normal morphology tightly attached to the culture dish, and only a few cells were stained in the nucleus or weakly in the cytoplasm. In the psiRNA-*C35*-1 and psiRNA-*C35*-2 transfected cells, concentrated and brown nucleus staining and lightly distributed cytoplasm staining was observed (Figure 5). This result showed that apoptotic cells in the psiRNA-*C35*-1 and psiRNA-*C35*-2 transfected groups were much greater than that of the Lipofectamine and pTZU6+1 transfected groups.

3.5. *Effect of psiRNA-C35 constructs on caspase-3 expression in T47D cells*

To investigate the mechanism by which siRNAs expressed by psiRNA-*C35* induces apoptosis of T47D cells, caspase activity was assessed by measuring the level of caspase-3 using Western blots. The results showed that expression of caspase-3 in the psiRNA-*C35*-1 and psiRNA-*C35*-2 transfected cells was much higher than that of the Lipofectamine and pTZU6+1 transfected cells (Figure 6). Also, the expression of caspase-3 in the psiRNA-*C35*-2 transfected cells was higher than that of the psiRNA-*C35*-1 transfected cells. The result suggested that siRNA expressed by psiRNA-*C35* might induce apoptosis of T47D cells by activating caspase-3. Further studies are needed to investigate the effect of psiRNA-*C35*, especially psiRNA-*C35*-2, on the

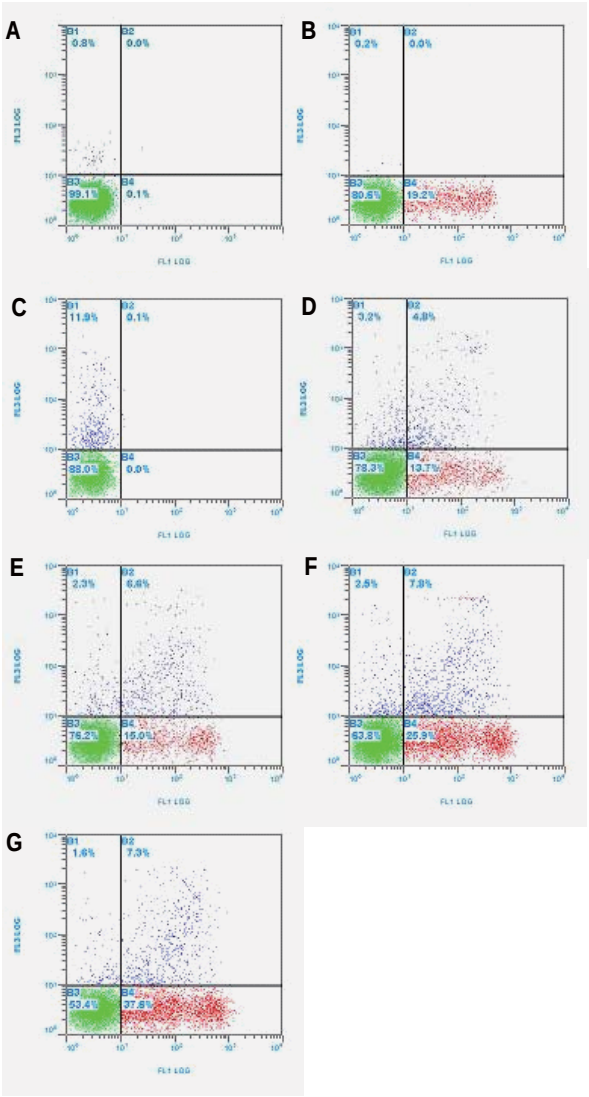

Figure 4. Effects of psiRNA-*C35*-1 and psiRNA-*C35*-2 on the apoptosis of T47D cells as determined by an Annexin V-FITC kit. A, T47D cells; **B**, only Annexin V; **C**, only PI; **D**, Annexin V + PI; **E**, pTZU6+1 + Annexin V + PI; **F**, psiRNA-*C35*-1 + Annexin V + PI; **G**, psiRNA-*C35*-2 + Annexin V + PI.

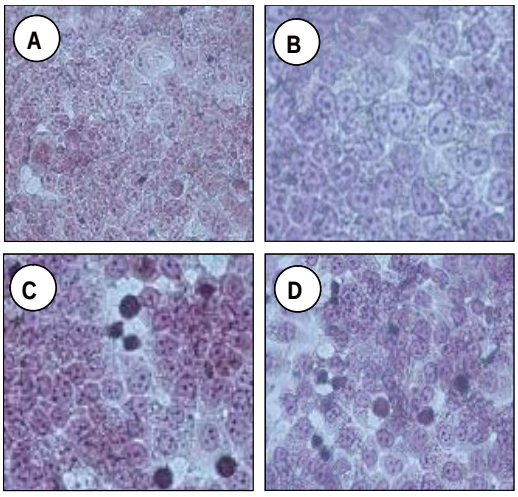

Figure 5. Effects of psiRNA-*C35*-1 and psiRNA-*C35*-2 on the apoptosis of T47D cells as determined by TUNEL assay. T47D cells were infected with a Lipofectamine control, pTZU6+1, psiRNA-*C35*-1, and psiRNA-*C35*-2 for 72 h. **A**, Lipofectamine control; **B**, pTZU6+1; **C**, psiRNA-*C35*-1; **D**, psiRNA-*C35*-2. Original Magnification: ×400.

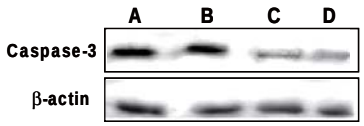

Figure 6. Western blot analysis of capase-3 expression in T47D cells infected with a Lipofectamine control, pTZU6+1, psiRNA-*C35*-1, and psiRNA-*C35*-2. A, Lipofectamine control; **B**, pTZU6+1; **C**, psiRNA-*C35*-1; **D**, psiRNA-*C35*-2.

expression of other apoptosis related molecules.

4. Discussion

Breast cancer has emerged as the most frequent malignant neoplasm in the world in recent years, raising awareness in society of the issue of breast cancer. Identification of cancer-specific biomarkers has enormous potential to enhance detection, treatment, and prognosis of breast cancer (*15*). In addition, understanding the role of such biomarkers in the process of transformation could reveal opportunities to target cancer-specific proteins therapeutically and increase treatment options for breast cancer.

C35, a newly reported biomarker for breast cancer, was found in overabundance in more than 60% of breast cancer cases (*5*). The gene is closely linked with a previously identified breast cancer gene, *HER2* (also spelled *HER2/neu*). *HER2* is a gene on the surface of cells that, when functioning normally, has been found to be a key component in regulating cell growth (*16,17*). It was shown that 34% of breast cancer patients were found to have an overabundance of both C35 and HER2, while 31% tested positive for C35 and negative for HER2. Interestingly, all of the breast cancer patients who over-expressed HER2 also over-expressed C35 (*5*). Overexpression of C35 in *HER2/neu*-negative breast cancer patients and breast tumor cell lines was confirmed, suggesting independent transcriptional control mechanisms for C35 (*5*). Thus, C35 might be a more effective biomarker for breast cancer, while the functional importance is presently unknown.

To explore the functional importance of C35, we constructed siRNAs targeting *C35* in this study. Due to its high efficiency and specificity, RNAi is now being widely used as a method to knockdown target genes, to study gene function or to be used in experimental treatment of some diseases (*18,19*). One problem in using siRNA to knockdown gene expression is target sequence selection. siRNAs that target different sites of the same gene can vary from strong to no inhibition of gene expression. In the present study, two siRNAs targeting different regions of the *C35* gene were chosen and their ability to silence the expression of the *C35*

was observed. The results showed that both mRNA and protein expression of *C35* were significantly inhibited by the two psiRNA-*C35* siRNAs, especially the psiRNA-*C35*-2, in T47D cells.

Annexin V-FITC and TUNEL assays showed that apoptosis was significantly induced when *C35* expression was silenced. These results suggested that inhibiting *C35* expression by siRNAs could significantly induce apoptosis of breast cancer cells. Since induction of apoptosis stimulates a cascade of events that ultimately leads to cell death, caspases are the main executioners of Fas-mediated apoptosis, irrespective of the ceramide signalling pathway (*20,21*). Caspase-3 is thought to be a key apoptotic "executioner" enzyme in mammalian cells because its activation triggers the cascade of enzymatic events that culminates in the death of the cells (*22*). Thus, we detected caspase activity by measuring the level of caspase-3 using Western blots in this study. The result showed that psiRNA-*C35* might induce apoptosis of T47D cells *via* activating caspase-3, which will be confirmed by further studies.

In conclusion, this study showed that apoptosis of T47D cells can be significantly induced by inhibiting *C35* expression using siRNAs, which may be caused by activating caspase-3. C35 might play an important role in apoptosis of breast cancer cells, and therapeutic strategies targeting *C35* may be useful for breast cancer treatment.

Acknowledgements

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Original Article

M_3 muscarinic receptors mediate acetylcholine-induced pulmonary vasodilation in pulmonary hypertension

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Summary **Information about the muscarinic receptor subtype(s) mediating pulmonary circulatory vasodilator responses to acetylcholine (ACh) is limited. The aim of this study was to pharmacologically characterize the muscarinic receptors associated with ACh-induced pulmonary vasodilation in a pulmonary hypertension model. Vasodilation of rabbit isolated buffer-perfused lungs in which pulmonary hypertension was induced with the thromboxane A_2 analogue U-46619 was evoked by ACh at a just maximally effective concentration (2×10^{-7} M). The effects of cumulative concentrations of three specific muscarinic receptor subtype antagonists [pirenzepine (M_1), methoctramine (M_2), and 4-diphenylacetoxy-*N*-methylpiperidine methiodide (4-DAMP, M_3)] on ACh-induced pulmonary vasodilation were determined. Double vascular occlusion pressure was recorded to locate the muscarinic receptors within the pulmonary vasculature. Based on the 50% inhibitory concentrations (IC_{50}), the rank of order of antagonist potency was 4-DAMP >> pirenzepine > methoctramine. The vascular effects of all three inhibitors were localized to the precapillary segment. These findings suggest that the vasodilator action of ACh on rabbit isolated perfused U-46619 pretreated lungs is mediated by M_3 muscarinic receptors located in the pulmonary arterial bed.**

Keywords: Pulmonary vasodilation, muscarinic receptors, rabbit

1. Introduction

Although acetylcholine (ACh) was the first endothelium-dependent vasorelaxant to be identified (*1*), the complex role of ACh in the regulation of pulmonary vascular tone remains unclear. Under normal resting conditions, ACh induces a vasopressor response of the pulmonary circulatory system, whereas during pulmonary hypertension ACh evokes a vasodilator response (*2-7*). In dogs with oleic acid-induced lung injury, vagotomy markedly increases the pulmonary arterial pressure (Ppa), thereby increasing pulmonary edema (*8*). Moreover, pancuronium bromide, which at clinically used doses is a potent M_2 and M_3 muscarinic receptor antagonist, increases Ppa in this model and this effect is potentiated by hypoxemia (*9*). These findings suggest that cholinergic vasodilation involving M_2 and/or M_3 receptors plays a role in the pulmonary hypertensive state. Limited information is available, however, about the muscarinic receptor subtype(s) mediating ACh-induced vasodilator responses of the pulmonary circulatory system (*10*). Although experiments on isolated pulmonary arteries indicate that ACh-induced vasodilation is mediated by M_3 receptors, the overall function of ACh in a whole lung preparation has not been elucidated (*11*). Furthermore, it is unknown whether the distinct muscarinic receptor subtypes mediating ACh-induced vasodilation are distributed heterogeneously within the pulmonary vasculature.

To pharmacologically characterize the muscarinic receptors associated with ACh-induced pulmonary vasodilation, we evaluated the effects of varying concentrations of specific muscarinic receptor subtype antagonists on ACh-induced vasodilation in a thromboxane A_2 analogue (U-46619)-stimulated pulmonary hypertension model. To localize muscarinic

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receptors within the pulmonary vasculature, double vascular occlusion pressure, a measure of the pulmonary capillary pressure, was recorded (*12-15*).

2. Methods

The protocol for this study was approved by The Tokyo University Laboratory Animal Care Committee. A standard rabbit perfused lung preparation *in vitro* (*2*,*11*,*16*) was modified as described below.

2.1. *Isolated perfused lung preparation*

Fifty-five New Zealand white rabbits of both sexes, weighing 2.5 to 3.5 kg, were anesthetized with sufficient volumes (4-6 mL) of pentobarbital sodium (50 mg/mL) administered through the marginal vein to produce surgical anesthesia. Local anesthesia of the neck was induced by infiltration with 0.5% lidocaine. A tracheostomy was performed and each rabbit was mechanically ventilated with room air at a rate of 30 to 40 breaths/min, a peak airway pressure of 10 to 12 cmH_2O, and a positive end-expiratory pressure of 2.5 cmH_2O. A catheter was inserted in the right jugular vein to administer drugs and to drain the lymph fluid during perfusion. Heparin (1,000 U/kg) was injected into the right jugular vein and additional pentobarbital sodium was injected before starting the pulmonary isolation procedure. After sternotomy, the pulmonary artery (PA) and left atrium (LA) were cannulated *via* the right and left ventricles, respectively, and the lungs were perfused slowly with blood-free Krebs-Henseleit solution [composition (in mM): NaCl 118; KCl 4.74; $CaCl_2 \cdot H_2O$ 2.54; KH_2PO_4 1.19; $NaHCO_3$ 26.2; pH 7.4] containing 3% bovine serum albumin at 37°C and ventilated with 95% air/5% CO_2 at a rate of 5 breaths/min, a tidal volume of 10 mL/kg, and a positive end-expiratory pressure of 2.5 cmH_2O.

To reduce the numbers of cells in the recirculating perfusates, the pulmonary circulatory system was initially flushed with 700 mL fresh perfusion solution at a rate of 20 to 40 mL/min. The lungs were then connected to a recirculatory perfusion system in which the perfusate in the venous reservoir was recirculated at a flow rate of 100 mL/min using a peristaltic pump (Masterflex pump) through a water-jacketed heating coil and then through a bubble trap into the pulmonary artery and back to the venous reservoir. The perfusate temperature was maintained at 37°C with a heated water bath and the total volume in the circuit was 200 mL.

The airway (Paw), pulmonary arterial (Ppa), and left atrial (Pla) pressures were monitored continuously and recorded using an 8-channel recorder (Nihon Kohden, Tokyo, Japan). Ppa and Pla were measured *via* ports in the respective cannulas. The vascular pressures were referenced to the mid-level of the LA and the venous reservoir height was adjusted to maintain the Pla at 4 mmHg, thereby maintaining the whole lung under West zone III conditions. The LA was wrapped loosely with gauze and glued to keep its volume constant. In the present study, we used only lungs that *i*) had a homogeneous white appearance with no signs of congestion, edema, or atelectasis and *ii*) maintained a constant pulmonary vascular pressure gradient (Ppa-Pla) of less than 7 mmHg when perfused at 100 mL/min and a peak ventilation pressure within the normal range. Indomethacin (30 μM) was included in the perfusate to reduce the vascular contractile response to ACh (*2*,*16*,*17*).

2.2. *Measurement of the pulmonary capillary pressure (Ppc)*

The Ppc was estimated using the double vascular occlusion method. Briefly, this method is based on the assumption that when the arterial inflow and venous outflow are occluded simultaneously, all vascular pressures are equal to the Ppc, because the majority of the pulmonary vascular compliance is attributable to the microcirculation (*12-15*). To measure the Ppc, the PA and LA cannulas were simultaneously occluded during the apneic period between ventilator breaths and during occlusion. The Ppa and Pla were digitized and sampled at 200 Hz using a Maclab A/D converter. From 2 to 3 s after double vascular occlusion, the difference between the Ppa and Pla was less than 0.5 mmHg, so their average was considered to represent the Ppc. The total vascular resistance (Rt) was determined by calculating the difference between the Ppa and Pla and dividing the result by the flow rate (Q). The precapillary resistance (Ra) was calculated as the difference between Ppa and Ppc divided by Q, and the postcapillary resistance (Rv) was calculated as the difference between Ppc and Pla divided by Q.

2.3. *Effects of ACh on the pulmonary vascular resistance under conditions of U-46619 induced pulmonary hypertension*

The lungs of 9 rabbits were initially perfused with Krebs-Henseleit solution containing 3% bovine serum albumin at 100 mL/min, as described above. To ensure strong stable vascular contraction, the Ppa was pharmacologically increased to 25 mmHg at Q = 50 mL/min providing that the lung preparation remained stable for 15 min (*i.e.*, constant Paw, Ppa, and Pla) after reducing Q. Baseline measurements were recorded. Pulmonary hypertension was induced by infusing the stable thromboxane A_2 analogue U-46619 into the venous reservoir (*6*,*7*,*16*). Initially, U-46619 was infused at 150 ng/min to achieve a Ppa of 25 to 26 mmHg and then the infusion rate was reduced to deliver an hourly dose equal to the initial total dose required

to achieve pulmonary hypertension (*18*). After a 20-min period of stable pulmonary hypertension, the Paw, Ppa, Pla, and Ppc (control values) were measured, after which a bolus of ACh was added to the reservoir every 6 min to produce final cumulative concentrations of 10^{-8}, 3×10^{-8}, 10^{-7}, 3×10^{-7}, and 10^{-6} M and the vasodilator responses were recorded 5 min after administering each bolus, because the maximal sustained effect was attained within 2 to 3 min.

2.4. *Effects of muscarinic receptor antagonists on ACh-induced pulmonary vasodilation*

Twenty-eight rabbits were randomly divided into 4 subgroups of 7 and their isolated lungs were perfused under baseline conditions and then with U-46619 to produce stable pulmonary hypertension, as described above. When the Ppa reading had been stable for 15 min, baseline Ppc, Ppa, Pla, and Paw values under conditions of U-46619 induced pulmonary hypertension were recorded and then a bolus of ACh was injected into the venous reservoir to produce a final concentration of 2×10^{-7} M. After a 20-min equilibration period, to confirm the stable decrease in Ppa induced by ACh, the Ppc, Ppa, Pla, and Paw were recorded, after which normal saline or the muscarinic receptor antagonist dissolved in normal saline was administered cumulatively at 6-min intervals, as described below, and Ppa, Pla, Ppc, and Paw were measured 5 min after each drug addition.

The magnitude of the ACh-elicited vasodilator responses of the preparations varied. Therefore, each Ra value is expressed as a ratio (B2/B1). Here, B1 is the decrease in Ra (Ra before saline or antagonist administration − Ra after administration of ACh). B2 is defined as increase of Ra after the administration of saline or antagonist.

2.5. *Four subgroups (n = 6) received the following treatment regimens*

Group 1 (saline alone, vehicle control) – Normal saline (3 mL) was injected into the venous reservoir 20, 26, 32, and 38 min after ACh administration and Ppa, Pla, Ppc, and Paw were recorded to assess the changes in pulmonary vascular resistance (PVR) during the course of the experiment.

Group 2 (selective M_1-receptor antagonist) – The M_1-receptor antagonist pirenzepine (2×10^{-8}, 10^{-7}, 5×10^{-7}, and 10^{-6} M) was administered cumulatively to assess the effects of M_1-receptor blockade on pulmonary vascular tone.

Group 3 (selective M_2-receptor antagonist) – The selective M_2-receptor antagonist methoctramine (10^{-7}, 5×10^{-7}, 2.5×10^{-6}, and 4×10^{-6} M) was administered cumulatively to assess the effect of M_2-receptor blockade on vascular tone after pulmonary vasodilation with ACh.

Group 4 (selective M_3-receptor antagonist) ─ The selective M_3-receptor antagonist 4-diphenylacetoxy-*N*-methylpiperidine methiodide (4-DAMP; 2×10^{-10}, 10^{-9}, 5×10^{-9}, and 10^{-8} M) was administered cumulatively to evaluate the effect of M_3 receptor blockade on the PVR after inducing pulmonary vasodilation with ACh.

2.6. *Drugs*

The drugs used in this study were all obtained from Sigma Chemical Co. (St. Louis, MO, USA).

2.7. *Statistical analysis*

The values are expressed as mean ± S.D. The statistical analysis was performed using one-way analysis of variance with repeated measures. For within-group comparisons, Student's *t* tests were used with Bonferroni's correction for multiple comparisons, as appropriate. Differences between means at $p < 0.05$ were considered significant.

3. Results

3.1. *Effects of ACh on the resistance of the pulmonary vasculature constricted with U-46619*

After recording the baseline pulmonary vascular pressures, rabbit isolated buffer-perfused lungs ($n = 9$) were treated with U-46619, followed by cumulative administration of ACh at 6-min intervals. U-46619 increased pulmonary vascular resistance (Rt) from 146.57 ± 12.76 to 421.71 ± 8.71 mmHg/L/min, with proportional increases in the right atrium (Ra) and right ventricle (Rv) (Figure 1). Under these conditions of elevated vascular tone, administration of ACh resulted in concentration-dependent decreases in Rt and Ra. The maximal vasodilatory effect was obtained with 10^{-7} M ACh and higher concentrations evoked no

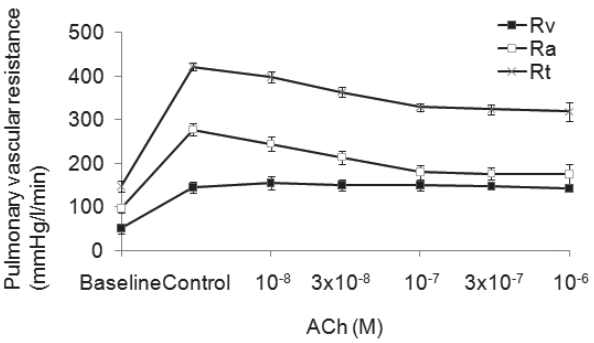

Figure 1. Effects of ACh (10^{-8}, 3×10^{-8}, 10^{-7}, 3×10^{-7}, and 10^{-6} M) on the pulmonary vascular resistance in rabbit isolated lungs pretreated with U-46619. * and # $p < 0.05$ *versus* baseline and control values, respectively. Values are mean ± S.D.

further decreases. In contrast, ACh did not alter Rv at any concentration tested, suggesting that ACh primarily induced precapillary vasodilation (*i.e.*, reduced Ra) without affecting the postcapillary vessels. ACh did not completely reverse U-46619-induced vasoconstriction and Rt and Ra after ACh administration were consequently higher than their baseline values ($p < 0.05$).

3.2. *Effects of muscarinic antagonists on ACh-induced pulmonary vasodilation*

Four injections of 3 mL saline at 6-min intervals had no effect on ACh-induced pulmonary vasodilation, which remained constant without changes in Rt, Ra, or Rv throughout the experiment. Therefore, a single bolus of ACh induced a persistent decrease in the PVR, which remained constant throughout the experiment with each antagonist.

The selective M_1- (pirenzepine), M_2- (methoctramine), and M_3- (4-DAMP) receptor antagonists all inhibited ACh-induced vasodilation in a concentration-dependent manner (Figures 2-4). Their inhibitory potencies, however, indicated by their different mean IC_{50} (the concentration of antagonist at which the control response was inhibited by 50%) values,

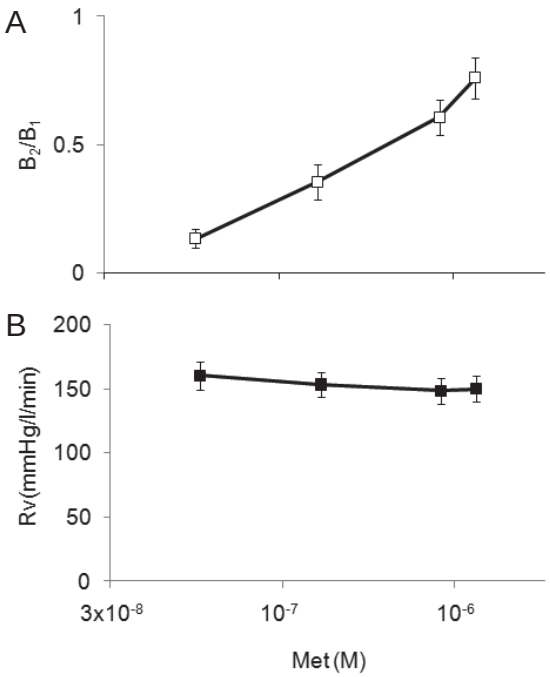

Figure 3. Effects of methoctramine (Met) on ACh-induced pulmonary vasodilation in the precapillary (A) and postcapillary (B) segments (*n* = 6). The results for the precapillary and postcapillary segments are expressed as mean B2/B1 ratios and mean absolute values, respectively. Vertical lines indicate mean ± S.D.

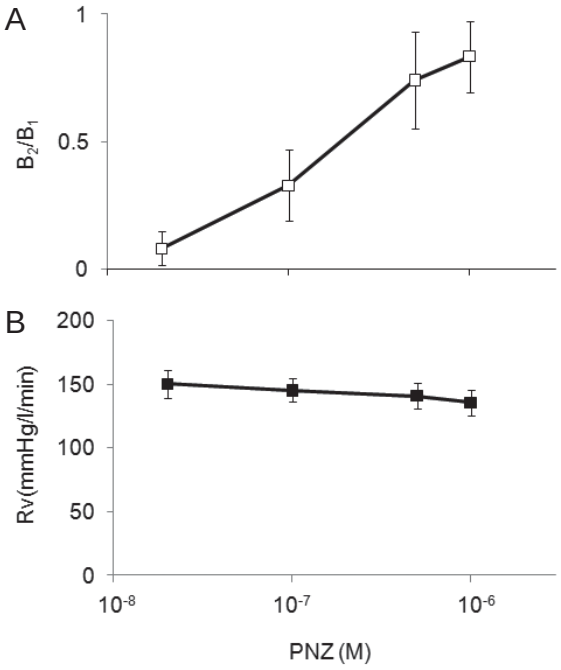

Figure 2. Effects of cumulative concentrations of pirenzepine (PNZ) on ACh-induced vasodilation of the precapillary (A) and postcapillary (B) segments of the rabbit perfused lung preparation (*n* = 6). The inhibitory effect of pirenzepine on the ACh-induced reduction in precapillary resistance is shown as an increase in the B2/B1 ratio [response after (B2) divided by that before (B1) exposure to pirenzepine]. The results for the precapillary and postcapillary segments are expressed as mean B2/B1 ratios and mean absolute values, respectively. Vertical lines indicate mean ± S.D.

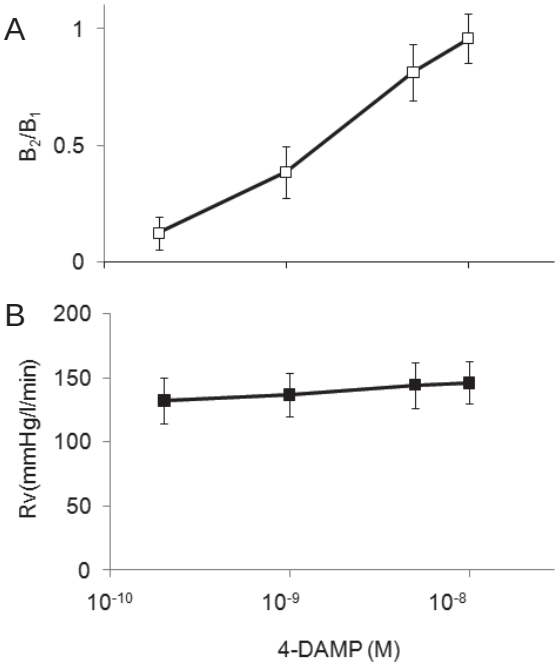

Figure 4. Effects of 4-DAMP on ACh-induced pulmonary vasodilation in the precapillary (A) and postcapillary (B) segments (*n* = 6). The results for the precapillary and postcapillary segments are expressed as mean B2/B1 ratios and mean absolute values, respectively. Vertical lines indicate mean ± S.D.

differed. Based on their IC_{50} values, the rank order of antagonist potency was 4-DAMP (1.58×10^{-9} M) >> pirenzepine (2.51×10^{-7} M) > methoctramine (1.25×10^{-6} M). Therefore, 4-DAMP was approximately 160 and 1,000 times more potent as an antagonist of ACh-induced pulmonary vasodilation than pirenzepine and methoctramine, respectively. It is noteworthy that the effects of all three antagonists were exerted exclusively on the precapillary segment, as none of them altered the Rv at the concentrations tested.

4. Discussion

The main finding of this study is that M_3-subtype muscarinic receptors mediated the vasodilator action of ACh on the pulmonary vascular bed of the rabbit whole lung preparation with induced pulmonary hypertension. Furthermore, ACh dilated the pulmonary arterial bed exclusively, which we attribute to the preferential distribution of M_3 receptors in the pulmonary arterial, rather than venous, bed. Functional integrity of the vascular tone may be necessary to activate the mechanisms governing regional blood flow and vascular resistance in the pulmonary circulatory system. Identification of pulmonary vascular muscarinic receptors is important toward understanding the process whereby ACh contributes to the regulation of pulmonary circulatory tone.

Muscarinic receptors are classified pharmacologically into five main subtypes: M_1, M_2, M_3, M_4, and M_5 (*19-22*). Differences between antagonist affinities are useful for differentiating receptor subtypes (*23*) and therefore the full concentration-response curves for a multiple set of selective muscarinic antagonists should be obtained, although this may not be easy to achieve in a perfused lung model. In the present study, three specific muscarinic subtype-receptor antagonists were used: pirenzepine, methoctramine, and 4-DAMP. Previous binding studies demonstrated that pirenzepine has high affinity for M_1 receptors and low affinity for M_2, M_3, M_4, and M_5 receptors (*10*,*22*,*24-26*). Methoctramine, a polymethylene tetraamine, is a more specific M_2-receptor antagonist and 4-DAMP has high affinity for M_3 receptors with a relatively low affinity for M_1 receptors (*10*,*22*,*24*,*25*). In the present study, pharmacodynamic characterization of muscarinic receptor subtypes was achieved by determining the effects of these three selective antagonists on the response to a just maximally effective concentration of ACh, determined from a concentration-response curve. Based on their IC_{50} values, the rank of order of antagonist potency was 4-DAMP (1.58×10^{-9} M) >> pirenzepine (2.51×10^{-7} M) > methoctramine (1.25×10^{-6} M). As M_3 receptors have a high affinity for 4-DAMP and a low affinity for pirenzepine (*22*), our findings show that ACh decreases the PVR in rabbit isolated lungs by activating M_3 receptors. ACh evokes a biphasic response in the rabbit pulmonary vascular bed (*2*,*3*,*5-7*) and selective abolition of ACh-induced contractile responses by inhibiting cyclooxygenase enables examination of the relaxant responses without functional interference from the opposing contractile response (*2*,*27*). Therefore, in this study, we used indomethacin to abolish the contractile response to ACh (*11*,*16*,*17*,*28*).

Studies of the muscarinic receptors that mediate ACh-induced pulmonary vasodilation have provided conflicting results. Several factors confound the available data, including interspecies differences, differences in the specific vascular bed under consideration, and the use of different experimental models. Our finding that the M_3 receptor is responsible for ACh-induced pulmonary vasodilation in a rabbit isolated perfused lung preparation extends the findings of studies on rabbit and feline lungs (*6*,*7*), which showed that the vasoconstrictor responses to ACh are blocked by pirenzepine, but neither pirenzepine nor galamine inhibits the vasodilator responses, suggesting that a third subtype of muscarinic receptor is responsible for the inhibitory effects.

Our results are also consistent with the findings of studies of isolated pulmonary arteries of several species: the selective M_3-receptor antagonist 4-DAMP inhibits ACh-induced relaxation of rat isolated pulmonary arterial strips at lower concentrations than does pirenzepine and methoctramine (*17*). A study on rabbit isolated pulmonary arteries also showed that 4-DAMP is a more potent inhibitor of the vasodilator response to ACh than pirenzepine or AF-DX116, a selective M_2-receptor antagonist (*10*). Our findings, however, conflict with the report that ACh-induced vasodilation in rat isolated perfused lungs preconstricted with U-46619 is mediated by M_1 receptors (*29*). Although the discrepant results may be partially due to the different species and experimental preparations used, only single high concentrations (μM) of the selective M_1 agonist and antagonist were used in that study, and therefore the possibility that muscarinic receptors other than M_1 were involved cannot be excluded.

In the present study, the double occlusion technique was used to partition the total pulmonary vascular resistance into segmental resistances (*12-14*). Consistent with previous observations, U-46619 significantly increased the PVR (represented by Rt) and both Ra and Rv (*18*). Under conditions of increased vascular tone, ACh reduced Ppa, Rt, and Ra, but had no effect on Rv at any concentration tested. These results demonstrate that ACh decreases the PVR of the pulmonary circulatory system of the rabbit solely by dilating the precapillary segment of the pulmonary vascular bed, which suggests an arterial predominance of vasodilatory muscarinic receptors. The selective M_3 antagonist 4-DAMP completely reversed ACh-induced vasodilation and the other antagonists,

which have much lower affinities for the M_3 receptor, inhibited ACh-induced vasodilation when much higher concentrations were used. The vascular effects of all three inhibitors were localized exclusively to the precapillary segment. These results strongly suggest that M_3 receptors are localized in the pulmonary arterial segment, although the exact anatomic distribution of the vasodilator muscarinic receptors in the lung remains to be determined.

We previously demonstrated that the pulmonary intravascular pressure in dogs with induced lung edema increases to a greater extent in dogs that are vagotomized than in those with normal innervation (*8*). Furthermore, the extravascular lung water content resulting from lung injury is greater in vagotomized than in normally innervated dogs. These findings suggest that vagal innervation plays an important role in controlling pulmonary vascular pressure under conditions of lung injury. Further, pancuronium, which at clinical doses is a potent M_2 and M_3 receptor antagonist, increases PAP and pulmonary vascular resistance, but does not affect systemic blood pressure and reduces the systemic vascular resistance in dogs with oleic acid-induced lung injury (*9*,*30*,*31*). These results suggest that the responses of the pulmonary and systemic vasculature to muscarinic inhibitors may be totally different under conditions of pulmonary hypertension and normotension and raise the possibility that cholinergic innervation and endogenous ACh play crucial roles in the physiology of the pulmonary vasculature.

In conclusion, these findings demonstrated that the vasodilator action of ACh in the isolated perfused rabbit lung pretreated with U-46619 is mediated mainly by M_3 muscarinic receptors. ACh dilates the pulmonary arterial bed, but not the venous bed, which is likely due to the preferential distribution of M_3 receptors in the pulmonary arterial bed.

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Original Article

Clinical benefits of two different dosing schedules of recombinant human erythropoietin in anemic patients with advanced head and neck cancer

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Summary **A total of 100 patients with stage III or IV head or neck cancer, a performance status of 0-1, and anemia with hemoglobin (Hb) < 10 g/dL at baseline who where to receive chemotherapy concomitantly or sequentially with radiotherapy were randomized to receive either epoetin beta 10,000 IU thrice weekly (TW) (n = 52) and oral iron starting 10-15 days before the start of treatment or epoetin beta 30,000 IU once weekly (OW) (n = 48) and oral iron before the start of treatment. The mean Hb in patients on the thrice weekly (11.96 g/dL) and once weekly (12.50 g/dL) dosing schedules increased significantly (p < 0.01) at the end of the treatment in comparison to respective baseline values of 9.38 g/dL and 9.41 g/dL; levels were 1.2-fold higher, which was significant (p < 0.01), for patients on the once weekly schedule. That said, there was significant improvement (p < 0.01) in mean linear analog scale assessment (LASA) scores for energy level (EL), ability to perform daily activities (AL), and overall quality of life (QOL) for patients on both dosing schedules but these improvements did not differ significantly between schedules (p > 0.05). The 2-year overall survival for patients on both dosing schedules did not differ significantly (p > 0.05). Epoetin beta therapy was found to be equally beneficial and well tolerated for patients on both thrice weekly and once weekly dosing schedules.**

Keywords: Head and neck cancer, anemia, epoetin beta, recombinant human erythropoietin, hemoglobin, quality of life (QOL)

1. Introduction

The worldwide incidence of head and neck cancers exceeds half a million cases and ranks such cancers as the 5th most common malignancies (*1*). New cases of such cancers total approximately 40,000 in the United States annually, accounting for 5% of all adult malignancies. On the Indian sub-continent, such cancers account for more than 25% of all malignancies due to use of cigarettes and chewing tobacco. More than 80% of head and neck malignancies present in locally advanced stages and have a poor prognosis; this has remained unchanged over the past 30 years. The 5-year survival rates of multimodal chemoradiotherapy are below 20%, with a median survival of 12 months or less (*2-4*). Therefore, palliation of symptoms and maintenance of quality of life (QOL) are primary goal of management (*5*).

Anemia is a well-recognized complication of stage IV head and neck cancer and its treatment adversely affects patients' well-being, QOL, and potential survival (*6-11*). Changes in chemotherapy, and particularly the introduction of new agents and treatment regimens, have increased the clinical significance of chemotherapy-related anemia (*9,10*).

Clinical data suggest that even mild to moderate chemotherapy induces anemia, resulting in a perceptible reduction in a patient's energy level, activity level, and overall QOL (*11-14*). Because of the infection and immunosuppressive risks associated with blood transfusions, as well as their transient effect

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on hemoglobin levels and amelioration of anemia, the use of erythropoietic agents has become the new standard of care for patients with chemotherapy-related anemia (*15*). Erythropoietic agents have been associated with decreases in transfusion requirements, higher transfusion-free survival, and significant improvements in QOL as measured by the Functional Assessment of Cancer Therapy-Anemia (FACT-An) and FACT-General (FACT-G) scales (*11*,*16-19*). Recently, erythropoietin has been found to be beneficial in correcting anemia in patients with carcinoma cervix treated with chemoradiotherapy (*20*).

Several randomized trials have compared a once weekly erythropoietin dosage regimen (30,000 IU *s.c.*) with a thrice weekly regimen (10,000 IU *s.c.*) in an effort to establish comparable efficacy between these schedules in patients with chemotherapy-related anemia (*21*,*22*). The investigators reported no significant differences between both groups with regard to changes in hemoglobin (Hb) levels, response rates, or blood transfusion requirements (*21*). The median time to a Hb increase of > 1 g/dL was similar for both the thrice weekly and once weekly regimens (*22*), so less frequent dosing schedules of erythropoietic agents appear to be as effective as the approved regimens.

2. Materials and Methods

2.1. *Patients*

A prospective study was conducted with a total of 100 histologically proven cases of stage III or IV head or neck cancer in patients in apparently good condition who were able to tolerate treatment (Grade 0 or 1 performance status on the World Health Organization (WHO) scale). The patients in question were thoroughly assessed (history, clinical examination, and investigations) and were scheduled to undergo Neoadjuvant 3-cycles paclitaxel and cisplatin chemotherapy at an interval of three weeks followed by concurrent chemoradiotherapy (planned concurrent cisplatin 35 mg/m^2 and conventional radiation dose of 70 Gy in 35 fractions over a period of 7 weeks in external therapy) and epoetin beta 10,000 IU 3 times a week or 30,000 IU once a week.

2.2. *Inclusion criteria*

Inclusion criteria for patients included having histologically proven stage III or IV head or neck cancer, a diagnosis of anemia, a life expectancy of more than 6 months, being in apparently good condition, being able to tolerate treatment, and a performance status of grade 0 or 1. Patients were not to have received any previous definitive treatment (radiotherapy, surgery, chemotherapy, *etc.*) for their disease. Patients with anemia secondary to vitamin deficiency, bleeding, or hemolysis, pregnant or nursing women, and patients undergoing stem-cell transplantation were excluded. Additional significant exclusion criteria were uncontrolled hypertension or cardiac arrhythmia, recent thromboembolism (unless the patient was receiving anticoagulation therapy), untreated brain metastases or seizures, and previous treatment with an erythropoietic agent in the previous 6 months, and anemia attributable to other factors (*e.g.* iron or folate deficiency, hemolysis, and gastrointestinal bleeding).

Eligible patients were randomized into two groups on a once weekly epoetin dosage regimen or on a thrice weekly epoetin dosage regimen; both groups receive epoetin beta along with definitive treatment. Patient randomization was done at a ratio of 1:1 at baseline by the sealed envelope method. This study was approved by this Institution's ethics committee and all participants provided written informed consent before entering the study.

2.3. *Treatment*

On the thrice weekly schedule, the starting epoetin dosage was 10,000 IU administered subcutaneously three times a week for a maximum of 16 weeks. The dosage of epoetin beta was increased to 20,000 IU three times a week if the increase in the Hb level was < 1.0 g/dL after 4 weeks of therapy. If, after an additional 4 weeks of therapy at the higher dosage level, the increase in the Hb level remained < 1.0 g/dL compared to the baseline, then epoetin beta therapy was discontinued. Patients were treated for a maximum of 16 weeks. Patients whose Hb levels exceeded 13 g/dL did not receive further epoetin beta treatment until these levels fell to 12 g/dL, at which time epoetin beta was resumed at 75% of the original dose and titrated to maintain the desired Hb level. The dose of epoetin beta was also reduced if Hb levels increased rapidly (*i.e.*, > 1.3 g/dL in any 2-week period). Patients were transfused based on clinical judgment and Hb < 10 g/dL.

Starting epoetin beta dosage was 30,000 IU administered once weekly by subcutaneous injection. If the increase in the Hb level was < 1.0 g/dL after 4 weeks of therapy, the epoetin beta dosage was increased to 60,000 IU once weekly. If the increase in Hb remained < 1.0 g/dL compared to the baseline after an additional 4 weeks of therapy at the higher dosage level, then epoetin beta therapy was discontinued. Patients were treated for a maximum of 16 weeks. Patients whose Hb levels exceeded 13 g/dL had epoetin beta treatment discontinued until their Hb level fell to 12 g/dL. Treatment with epoetin beta was resumed at 75% of the previous dose and titrated to maintain the desired Hb level. The dose of epoetin beta was also reduced if Hb levels increased rapidly (> 1.3 g/dL in any 2-week period). Patients were transfused based

on clinical judgment and Hb < 10 g/dL. All patients were randomly assigned immediately upon enrollment and were scheduled to receive epoetin over a 16-week treatment period. The drug was administered by a health care provider; self-injection was not permitted, and dose modification was done depending on the Hb level.

2.4. *Evaluations*

Baseline information included patient demographics, weight and blood pressure, tumor histology, current chemotherapy and radiation therapy regimens, Hb level, and transfusion use. Patients were seen and evaluated each month until the completion of treatment. Monthly evaluations included the Hb level, maintenance of the Hb level, transfusion requirements since the last study visit, adverse events, and improvement in overall QOL, which was rated by patients using a 100-mm linear analog scale assessment (LASA) that rated energy level, ability to perform daily activities, and overall QOL. LASA assessment done by placing vertical marks on each line of a 100-mm linear analog scale to indicate the patient's answers regarding his or her energy level, ability to perform daily activities, *i.e.*, activity level, and overall QOL. LASA was performed at baseline, *i.e.*, before starting the first cycle of chemotherapy. Improvements in LASA were assessed gradually at the completion of each cycle of chemotherapy and during and at the completion of chemoradiotherapy for both groups of patients on the thrice weekly and once weekly epoetin dosage regimens. Tumor response was classified according to the WHO criteria (*23*). Adverse events were monitored and graded using the National Cancer Institute Common Toxicity Criteria version 2.0 (*24*).

2.5. *Statistics*

Changes in the mean levels of Hb, energy level, activity level, and overall QOL from baseline to the end of treatment for patients on both schedules were assessed with a two sample paired *t*-test while the mean differences in those indices for patients on the two schedules were determined with a two sample unpaired *t*-test. Categorical data were compared with Fisher's exact test, a χ^2 test, and a proportion Z-test. Cumulative survival rates for patients on both schedules were calculated using Kaplan-Meier's method and the difference between survival rates was evaluated with a log-rank test. The power of the effect size was evaluated according to Cohen (*25*). The power of the effect size, as measured by a two-sided *t* test with a 5% type I error rate, was predicted to have 80% power to detect a difference of 0.29 in Hb for patients on the two dosing schedules with a 95% CI for 95 subjects in total.

A two-tailed ($\alpha = 2$) probability value of $p < 0.05$ was considered to be significant. MS EXCEL (MS Office 1997-2003) and GraphPad Prism (version 5) were used for analysis.

3. Results

One hundred patients in total were enrolled in the study, of which 52 were randomly selected to receive epoetin beta 10,000 IU thrice weekly and 48 were randomly selected to receive epoetin beta 30,000 IU once weekly; both groups received epoetin beta along with definitive treatment. The once weekly dosage group had 45 evaluable patients in total (3 patients withdrew before the start of treatment) and the thrice weekly dosage group had 50 such patients (2 patient withdrew before the start of treatment).

The baseline characteristics of subjects on the two dosing schedules are summarized in Table 1. Comparison indicated that the baseline characteristics for patients on the two dosing schedules did not differ significantly, *i.e.*, statistically they were found to be the same.

The levels of Hb, energy level, activity level, and overall QOL at baseline, at the end of the treatment, and changes from baseline to end of treatment for patients on the two dosing schedules are summarized in Table 2. At baseline, at the end of treatment, and changes in the mean level of Hb, energy level, activity level, and overall QOL did not differ significantly ($p > 0.05$) for patients on the two dosing schedules, except for changes in Hb, which were significantly higher ($p < 0.01$) for patients on the once weekly schedule (Table 2). The changes (improvement) in Hb, energy level, activity level, and overall QOL for patients on both dosing schedules were found to be significantly higher ($p < 0.01$) at end of the treatment in comparison to the respective baseline values (Table 2).

The improvement in Hb, energy level, activity level, and overall QOL for patients on the two dosing schedules is summarized in Table 3. The improvement in Hb, energy level, activity level, and overall QOL for patients on the thrice weekly dosing schedule was found to be 2.57, 40.26, 30.29, and 23.84 g/dL, respectively, while the improvement for patients on the once weekly dosing schedule was 3.13, 40.68, 30.63,

Table 1. Baseline characteristics of patients on two different dosing schedules

Characteristics	Once weekly	Thrice weekly
Total patients registered (*n*)	48	52
Mean age (yrs)	48.18 (45-72)	48.27 (43-70)
Histology		
Squamous	46	49
Unspecified	2	3
Stage		
III	24	25
IV	24	27
Mean hemoglobin (g/dL)	9.41 (9-10)	9.38 (9-10)

Values in parentheses indicate the range.

Table 2. Statistics (Mean ± S.D.) on outcome variables for patients on two different dosing schedules

Variables	Treatments	Initial (baseline)	Final (end of CRT)	Change (Final – Initial)
Hb	OW	$9.41 \pm 0.43_{(48)}$	$12.50 \pm 0.49_{(45)}$	$3.13 \pm 0.41_{(45)}^{**}$
	TW	$9.38 \pm 0.39_{(52)}$	$11.96 \pm 0.46_{(50)}$	$2.57 \pm 0.53_{(50)}^{**}$
	t (OW *vs.* TW)	0.26^{ns}	5.59^{**}	5.67^{**}
EL	OW	$34.50 \pm 0.71_{(48)}$	$75.18 \pm 0.75_{(45)}$	$40.68 \pm 0.83_{(45)}^{**}$
	TW	$34.15 \pm 1.78_{(52)}$	$74.46 \pm 2.71_{(50)}$	$40.26 \pm 3.39_{(50)}^{**}$
	t (OW *vs.* TW)	1.26^{ns}	1.72^{ns}	0.81^{ns}
AL	OW	$36.55 \pm 0.72_{(48)}$	$67.11 \pm 0.71_{(45)}$	$30.63 \pm 0.88_{(45)}^{**}$
	TW	$36.38 \pm 0.69_{(52)}$	$66.64 \pm 1.91_{(50)}$	$30.29 \pm 1.93_{(50)}^{**}$
	t (OW *vs.* TW)	1.26^{ns}	1.56^{ns}	1.09^{ns}
QOL	OW	$42.19 \pm 0.73_{(48)}$	$66.44 \pm 0.62_{(45)}$	$24.30 \pm 0.78_{(45)}^{**}$
	TW	$42.10 \pm 1.50_{(52)}$	$65.94 \pm 2.00_{(50)}$	$23.84 \pm 2.48_{(50)}^{**}$
	t (OW *vs.* TW)	0.38^{ns}	1.62^{ns}	1.19^{ns}

^{ns} $p > 0.05$; ** $p < 0.01$; Data expressed as mean ± S.D. The numbers in parentheses indicate the number of patients.
Abbreviations: Hb, hemoglobin; EL, energy level; AL, activity level; QOL, overall quality of life; OW, once weekly; TW, thrice weekly; CRT, chemoradiotherapy.

and 24.30 g/dL, respectively. The net improvement in Hb, energy level, activity level, and overall QOL was respectively 1.2, 1.0, 1.0, and 1.0-fold higher for patients on the once weekly schedule, but none of these improvements were statistically significant ($p > 0.05$) except for improvement in Hb.

The mean time to an increase in Hb of > 1 g/dL was 4.2 and 4.4 weeks for patients on the thrice weekly and once weekly dosage regimen, respectively, although the difference was not statistically significant. The mean target Hb level of > 12 g/dL and maintenance of Hb between 11.96 g/dL to 12.50 g/dL was achieved in 64.9% and 65.5% of the thrice weekly and once weekly group, respectively.

The response rate, 2-year survival, disease-free survival, and reduction in blood transfusions for patients on the two dosing schedules are summarized in Table 4. The number of blood transfusions did not differ significantly ($p > 0.05$) for patients on a thrice weekly (54%) or once weekly (53%) schedule. Similarly, the reduction in blood transfusions did not differ significantly ($p > 0.05$) for patients on a once weekly (46%) and thrice weekly (46%) schedule. The one-month overall response rate, 2-year overall median survival (Figure 1), disease-free survival, and survival rate for patients on the two dosing schedules did not differ significantly, either ($p > 0.05$). The incidence of acute toxicity was similar for patients on both treatment schedules (Table 5). There were no adverse events related to epoetin beta in either of the treatment groups.

4. Discussion

Despite the potential benefits of erythropoietic agents in cancer-associated anemia, less than one half of eligible persons currently receive therapy with these drugs (*26*). Many factors may contribute to this putative underuse, including cost factors and US Food and Drug administration-approved schedules, as frequent doses

Table 3. Improvement (% mean change) in outcome variables for patients on two different dosing schedules

Variables	Once weekly (%)	Thrice weekly (%)	Net improvement (Once weekly/Thrice weekly)
Hb	32.9	27.4	1.2^{*}
EL	117.9	118.0	1.0^{ns}
AL	83.6	83.2	1.0^{ns}
QOL	57.5	56.6	1.0^{ns}

Abbreviations: Hb, hemoglobin; EL, energy level; AL, activity level; QOL, overall quality of life; OW, once weekly; TW, thrice weekly; ^{ns} $p > 0.05$; * $p < 0.05$.

Table 4. Response rate and prognosis for patients on two different dosing schedules

Characteristics	Once weekly	Thrice weekly
Total patients evaluated (*n*)	45	50
Blood transfusion (*n*)	24	27
One-month overall response rate (%)	93	96
2-year overall median survival (months)	23	20
2-year disease-free survival (%)	60	62
2-year survival rate (%)	52	58
Reduction in blood transfusions (%)	46	46

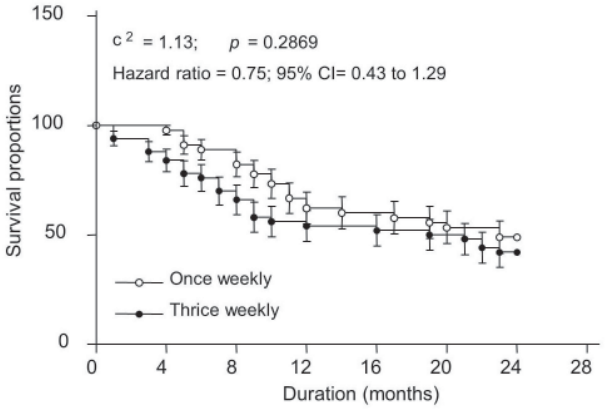

Figure 1. Cumulative survival proportions (%) for patients on two different dosing schedules with standard error (vertical bar).

Table 5. Grade-wise acute and late toxicity (%) for patients on two different dosing schedules

Toxicity	Once weekly				Thrice weekly			
	Grade 1 toxicity (G1)	Grade 2 toxicity (G2)	Grade 3 toxicity (G3)	Grade 4 toxicity (G4)	Grade 1 toxicity (G1)	Grade 2 toxicity (G2)	Grade 3 toxicity (G3)	Grade 4 toxicity (G4)
Acute								
Skin	100	94	30	0	100	92	32	0
Mucositis	100	98	39	4	100	98	40	4
Stomatitis	100	98	35	4	100	97	45	4
Chewing and Eating	100	98	69	3	100	98	69	4
Xerostomia	95	97	45	5	96	96	39	4
Dysphagia	79	69	26	4	79	69	26	4
Dysgeusia	93	59	32	0	90	56	30	0
Anorexia	85.6	86.7	35	4	86	87	34	4
Nausea	87	85	36	3	87	85	34	4
Hematological	20	18	8	0	22	16	7	0
Infection	15	10	2	0	14	8	1	0
Late								
Radiation necrosis	2	1	1	1	2	2	1	0
Chronic xerostomia	76	73	42	4	75	70	30	3
Loss of taste	49	33	29	0	49	30	29	0
Tooth decay	30	28	4	0	29	26	3	0

are required to get the desired results, *e.g.* in a recent summary of trials including patients with solid tumors, epoetin beta (100-200 IU/kg *s.c.* thrice weekly) elicited mean Hb increases of 0.89-2.7 g/dL from baseline to last assessment, in durations ranging from 8 to 24 weeks (*16*).

Epoetin beta is also associated with decreases in transfusion requirements of 37-90% compared with no treatment (*16*). In a randomized, double-blind, placebo-controlled trial that enrolled 349 patients with transfusion-dependent hematologic malignancies, epoetin beta 150 IU/kg *s.c.* thrice weekly improved hematologic parameters and QOL (*19*). Since a thrice weekly dosage schedule is inconvenient for the patient and physician alike, a once weekly epoetin beta dosage was recently compared with the thrice weekly regimen in an effort to establish comparable efficacy between these schedules in patients with chemotherapy-related anemia.

In an open-label trial, 241 patients with lymphoproliferative malignancies with baseline Hb 9-11 g/dL and low serum erythropoietin levels (< 100 Mu/mL) were randomly selected to receive epoetin beta 10,000 IU *s.c.* thrice weekly or 30,000 IU *s.c.* once weekly (*21*). The investigators reported no significant differences between groups regarding changes in Hb levels, response rates, or blood transfusion requirements (*21*). The median time to Hb increase of > 1 g/dL was similar for patients on both the thrice weekly and once weekly regimens (*22*). The authors concluded that epoetin beta administered once weekly is an effective and convenient treatment for anemia in patients with lymphoproliferative malignancies and defective endogenous erythropoietin production (*21*).

The results of the current study are very similar to those reported in the literature indicating that once weekly and thrice weekly regimens of epoetin beta have comparable efficacy, *i.e.*, the increase in Hb levels, decreased transfusion requirements, improved functional status, overall QOL, and safety profiles of the thrice weekly and once weekly dosing schedules appear to be similar for anemic patients with cancer of the head and neck who were receiving radiotherapy concomitantly or sequentially with chemotherapy. In addition, a once weekly dosing schedule is more convenient for patients and physicians alike.

5. Conclusion

Erythropoietic agents have a well-established efficacy for treating chemoradiotherapy-related anemia in head and neck cancer, leading to statistically significant improvements in hematologic parameters, QOL, and decreased transfusion requirements. Once weekly and thrice weekly epoetin beta regimens exhibit similar efficacy and safety profiles in patients with head and neck cancer who are receiving chemoradiotherapy. Therefore, a once weekly dosage regimen appears to be as effective as approved regimens and offer greater convenience for patients and clinicians.

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Original Article

Efficacy of granulocyte colony stimulating factor as a secondary prophylaxis along with full-dose chemotherapy following a prior cycle of febrile neutropenia

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Summary **Secondary prophylaxis with recombinant human granulocyte colony stimulating factor (G-CSF) is recommended where patients have experienced febrile neutropenia in an earlier chemotherapy cycle and for whom the maintenance of chemotherapy dose intensity is important; or where febrile neutropenia has not occurred but prolonged neutropenia is causing excessive dose delay or reduction, where maintenance of dose intensity is important. The objective of this study was to determine the efficacy and feasibility of G-CSF as secondary prophylaxis when used along with full dose moderately myelotoxic chemotherapy following a prior cycle with febrile-neutropenia. Fifty-two patients aged 22-75 years with febrile neutropenia that required intravenous antibiotics following moderately myelotoxic chemotherapy were included. These patients received the next cycle of the same chemotherapy regime without dose modification but with support of filgrastim 24 h after completion of chemotherapy (300 µg/day/subcutaneously (*s.c.*) for weight < 60 kg, 480 µg/day/*s.c.* for weight > 60 kg, for at least 10 consecutive days), patients in whom neutropenia was associated with a life-threatening infection and those who developed prolonged myelosuppression were excluded. The use of the hematopoietic growth factor G-CSF was shown to shorten the neutrophil recovery time, resulting in significant reduction of incidence of febrile neutropenia, hospitalization and use of broad spectrum antibiotics. There was no drug related death or adverse events associated with either cycle. In conclusion, recombinant human G-CSF is effective and relatively safe as a secondary prophylaxis with full dose chemotherapy in patients who develop febrile neutropenia following prior cycles of moderately myelotoxic chemotherapy.**

Keywords: Febrile neutropenia, G-CSF, toxicity, chemotherapy, antibiotics

1. Introduction

Association of neutropenia and infection continues to be a major cause of morbidity and mortality in cancer patients receiving myelosuppressive chemotherapy (*1*). Prompt initiation of empiric broad-spectrum antibiotics has effectively improved the outcome of patients with febrile neutropenia. However, a substantial proportion of these patients require prolonged hospital stays or develop more severe medical complications.

Febrile neutropenia is defined as an absolute neutrophil count (ANC) of $< 0.5 \times 10^9$/L and an oral temperature of > 38°C and is a serious consequence of chemotherapy-induced neutropenia (CIN), frequently resulting in hospitalization, infectious complications, the use of intravenous (*i.v.*) antibiotics, chemotherapy dose delays or reductions, reduced treatment effectiveness, increased health care expenditures, reduced quality of life, and possibly death (*2-9*).

Hematopoietic colony-stimulating factor (CSF), such as granulocyte CSF (G-CSF) has shown to promote proliferation, differentiation, and function of

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progenitor and mature cells of the myeloid lineage (*10*). These cytokines also stimulate the bactericidal functions of mature neutrophils (*11*). When administered as a preventive adjunct to chemotherapy, CSFs have shown in clinical trials to shorten the neutropenic period and to reduce by 50% the incidence of febrile neutropenia in high-risk patients (*3,7,12*).

A recent meta-analysis of randomized controlled trials evaluating the treatment of febrile neutropenia with G-CSFs plus antibacterials *vs.* antibacterials alone demonstrated a significant reduction in the proportion of patients with prolonged hospitalization among those who received G-CSFs (*13,14*). The occurrence of neutropenia can lead to a delay in subsequent cycles of chemotherapy or a reduction in the doses of drugs in the regimen, which can compromise the efficacy of the chemotherapy (*4,5*). In aggressive non-Hodgkin's lymphoma (NHL) and early-stage breast cancer, there are data suggesting that the administration of a chemotherapy planned dose on time is associated with improved outcomes (*5-9,15-18*).

Several recent studies have shown that many patients, particularly older individuals, are routinely given both lower planned and delivered chemotherapy doses than the standard reference regimens (*15-18*). However, clinical trial data show that older patients can have outcomes (overall survival and disease free survival) similar to those of younger patients when adequate (*i.e.*, literature-cited, standard-dose) chemotherapy dose intensity is delivered (*19-21*). Furthermore, it has been suggested that older patients are more susceptible to myelotoxicity, possibly because of decreased hematopoietic reserves (*22,23*). The National Comprehensive Cancer Network (NCCN), USA, recently published guidelines recommending that patients aged 70 years and over who are treated with moderately toxic regimens should be treated prophylactically with hematopoietic growth factors (HGFs) to reduce myelotoxicity of the chemotherapy planned dose on time (*23*). Therefore CIN is clearly a serious consequence of myelosuppressive chemotherapy, but it can be managed successfully.

The optimal strategy is to prevent occurrence of neutropenia, and in patients with an increased risk of developing chemotherapy-related infections, prophylactic administration of CSFs may be warranted (*3*). Published data clearly establish that the optimal use of filgrastim requires initiation 24 to 72 h after the completion of the chemotherapy (*3*) and that delaying the start of filgrastim until the time of the ANC nadir is less effective. Furthermore, the administration of G-CSF over all cycles of chemotherapy leads to a cumulative benefit in terms of reduced incidence and duration of severe neutropenia in later cycles compared with cycle 1. This is thought to be due to a priming effect of G-CSF on neutrophil recovery by enhancing cell differentiation of the post-mitotic pool (*7*).

This study aimed to evaluate the efficacy and feasibility of G-CSF as a secondary prophylaxis when used along with full dose moderately myelotoxic chemotherapy following a prior cycle with febrile-neutropenia in solid tumors.

2. Materials and Methods

2.1. *Patients*

Between January 2003 and December 2008, 52 patients with febrile neutropenia that required intravenous antibiotics following moderately myelotoxic chemotherapy were included. The age of patients was in a range of 22-75 years (median 47). These patients received the next cycle of the same chemotherapy regime without dose modification but with support of filgrastim (300 μg/day/*s.c.* for weight < 60 kg, 480 μg/day/*s.c.* for weight > 60 kg, for at least 10 consecutive days) if the following criteria were fulfilled: age 18-70 years, WHO performance status ≤ 2, febrile neutropenia during last cycle not associated with septicemia or other life-threatening infection; complete recovery of neutrophils (> 1,500/mm^3) and platelets (> 100,000/mm^3) on the first day of the following cycle and the last chemotherapy cycle not associated with dose-limiting toxicity other than febrile neutropenia. Patients in whom neutropenia was associated with a life-threatening infection and those who developed prolonged myelosuppression were excluded. Diagnoses included lymphoma (n = 22), breast cancer (n = 21), germ cell tumor (n = 3), or non-small cell lung cancer (n = 6). Filgrastim (300 mg/day) was given subcutaneously starting 24-30 h after the last chemotherapy dose. A total of 8-9 alternate day doses were given routinely. However, if the absolute neutrophil count did not reach 1,500/mm^3 after the neutrophil nadir, G-CSF was given for a longer period. Subsequent cycles were given with filgrastim support and without dose reductions if no other dose limiting toxicity developed. Data concerning the incidence of febrile neutropenia, infection, and other dose-limiting toxicities that developed during the first four cycles given with secondary G-CSF prophylaxis were analyzed.

All patients signed a standard informed consent form before the start of each chemotherapy regimen.

2.2. *Study end points*

Primary end point was the duration of hospital stay. Secondary end points were, days on antibiotic therapy, incidence of fever, time to resolve fever, dose reduction, dose delay, and any incidence of adverse events in successive cycles of chemotherapy.

2.3. *Statistical analysis*

Groups (cycles) were compared using non parametric Friedman one way analysis of variance (ANOVA) by ranks followed by Dunn's multiple comparison test. Rank correlation was used to calculate relative association among the measures (variables). Proportions were compared by χ^2 and proportion Z-test. A two-tailed ($\alpha = 2$), probability (p) value less than 0.05 ($p < 0.05$) was considered to be statistically significant. MS EXCEL (MS Office 97-2003) and GraphPad Prism (version 5) were used for the analysis.

Table 1. Characteristics of patients ($n = 52$) treated with prophylactic G-CSF

Characteristics/Diagnosis/Treatment	Number of Patients
Characteristics	
Median age in yrs (range)	47 (22-75)
Diagnosis	
Lymphoma number (%)	22 (42.31%)
Breast cancer number (%)	21 (40.38%)
Testicular germ cell tumor number (%)	3 (5.77%)
Non small cell lung cancer number (%)	6 (11.54%)
Treatment	
CHOP/CHOP like number	18
COPP	2
ABVD	2
PEB	3
Cisp/Etop	6

3. Results

3.1. *Patients and chemotherapy regimens*

Fifty-two consecutive patients who fulfilled the study criteria were treated between January 2003 and December 2008. The major characteristics, diagnosis and their treatment regimens associated with neutropenic fever are shown in Table 1. As can be seen, the most common diagnosis was lymphoma (42%) and breast cancer (40%). More than half (58%) received adriamycin-containing regimens. Chemotherapy was given as adjuvant or neoadjuvant therapy.

Two patients in whom neutropenia was associated with a life-threatening infection and those who developed prolonged myelosuppression during the treatment were excluded from the study. Thus, efficacy of prophylactic G-CSF was investigated on 50 patients and evaluated statistically.

3.2. *End measures*

The end measures such as neutrophil recovery time, duration of fever, duration of antibiotic and duration of hospitalization are summarized graphically in Figure 1. Figure 1 showed that all these measures decrease with the progression of cycles. Inter comparison of

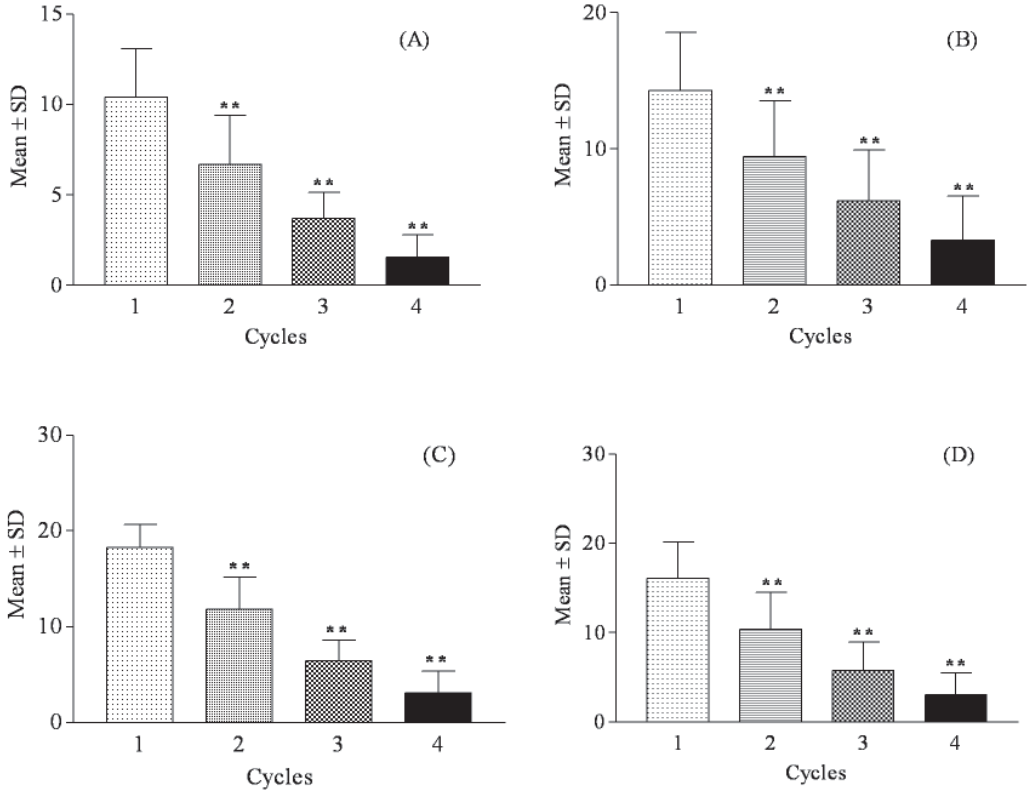

Figure 1. Cycle wise trend of neutrophil recovery time (A), duration of fever (B), duration of antibiotic (C), and duration of hospitalization (D) of patients treated with prophylactic G-CSF. ** $p < 0.01$.

Table 2. End measures at 4 cycles of patients (n = 50) treated with prophylactic G-CSF

Measures	Cycles				χ^2 (DF = 12)
	1	2	3	4	
Incidence of febrile neutropenia	32	9	5	2	4.37^{ns}
Incidence of antibiotic	32	9	5	2	
Incidence of antifungal	6	1	0	0	
Cycle delay	12	4	1	0	
Dose reduction	13	6	2	0	

Data represents number of patients. $^{ns}p > 0.05$.

Table 3. Inter-correlation (n = 200) among variables

Variables	Cycles	Neutrophil recovery time	Duration of fever	Duration of antibiotic	Duration of hospitalization	Incidence of fever	Incidence of antibiotic	Incidence of antifungal	Cycle delay	Dose reduction
Cycles	1.00									
Neutrophil recovery time	−0.90*	1.00								
Duration of fever	−0.52*	0.58*	1.00							
Duration of antibiotic	−0.53*	0.57*	0.97*	1.00						
Duration of hospitalization	−0.53*	0.57*	0.97*	1.00*	1.00					
Incidence of fever	−0.49*	0.56*	0.97*	0.92*	0.92*	1.00				
Incidence of antibiotic	−0.49*	0.52*	0.95*	0.99*	0.99*	0.92*	1.00			
Incidence of antifungal	−0.23*	0.24*	0.25*	0.26*	0.25*	0.21*	0.21*	1.00		
Cycle delay	−0.31*	0.35*	0.43*	0.44*	0.44*	0.37*	0.37*	0.43*	1.00	
Dose reduction	−0.31*	0.34*	0.56*	0.54*	0.54*	0.53*	0.49*	0.29*	0.60*	1.00

* $p < 0.01$

groups (cycle) showed the median (or mean) values of all these measures decreased significantly ($p < 0.01$) in cycle 2, 3, and 4 as compared to cycle 1 (Figure 1) while the levels of all these did not differ significantly ($p > 0.05$) in cycle 2, 3, and 4 except neutrophil recovery time. The neutrophil recovery time in cycle 3 and 4 also decreased significantly ($p < 0.05$) from cycle 2, while the level in cycle 3 and 4 did not differ significantly ($p > 0.05$) *i.e.*, were found to be statistically the same.

The other end measures such as incidence of fever, antibiotic, antifungal, cycle delay and dose reduction in patients are summarized in Table 2. Table 2 showed that as cycles increased the incidence of these measures in patients decreased. When compared the decrease in all measures over the cycles were found to be insignificant ($\chi^2 = 4.37$, $p > 0.05$).

The inter-correlation of all the above end measures over cycles (1 to 4) is summarized in Table 3. All measures showed a significant ($p < 0.01$) and inverse (negative value) correlation with the progression of cycles *i.e.*, as number of cycles increases, the incidence of symptoms decreases while the correlation among measures were found to be positive and significant ($p < 0.01$).

The initial and final evaluations of adverse events in patients are summarized in Table 4. All adverse events in patients decreased significantly ($p < 0.05$ or $p < 0.01$) in the final evaluation as compared to the initial except, diarrhea, musculoskeletal pain (severe, moderate, mild), and Grade III mucositis/stomatitis.

Table 4. Adverse events in patients (n = 50) treated with prophylactic G-CSF

Adverse events	Initial (n)	Final (n)	Z-test
Vomiting grade III	15	3	2.86**
Fatigue	13	2	2.80**
Diarrhea	7	3	1.00^{ns}
Anemia	10	1	2.56*
Fever	32	2	6.12**
Musculoskeletal pain	19	Same	No change
Severe	6	Same	No change
Moderate	6	Same	No change
Mild	7	Same	No change
Weakness	30	2	5.79**
Dizziness	20	1	4.42**
Grade III mucositis/stomatitis	7	1	1.84^{ns}

$^{ns}p > 0.05$; * $p < 0.05$; ** $p < 0.01$.

4. Discussion

The American society of clinical oncology (ASCO-2005) and ASCO-2005 update guidelines support the use of secondary prophylactic G-CSF in patients who had experienced a neutropenic complication (febrile neutropenia or prolonged neutropenia) from a prior cycle of chemotherapy (for which primary prophylaxis was not received), in which a reduced dose may compromise disease free or overall survival or treatment outcome. The ASCO-2005 update gives evidence that G-CSF recipients experienced fewer episodes of hospitalization for febrile neutropenia and

greater dose-intensity compared to historical controls *i.e.*, CSF support, but none of the other significant clinical outcomes (survival, quality of life, toxicity or cost) were reported (*1*).

Several relevant studies have been reported since 2000. In a multicenter trial conducted in Spain, adult patients with solid tumors or lymphoma who developed febrile neutropenia and had at least one high-risk factor, were treated with intravenous antibiotics and randomly assigned to receive G-CSF (5 μg/kg per day) until neutrophil recovery. CSF recipients had a shorter period of grade 4 neutropenia (median 2 *vs.* 3 days, $p = 0.0004$), antibiotic therapy (median 5 *vs.* 6 days, $p = 0.013$), and hospital stay (median 5 *vs.* 7 days, $p = 0.015$) (*2*). Survival between groups was similar.

In a Cochrane systematic review and meta-analysis, which included 1,518 patients from 13 trials, patients randomized to receive CSF experienced less prolonged neutropenia [25% *vs.* 45%; OR = 0.32 (0.23-0.46); $p < 0.00001$], less prolonged hospitalization [23% *vs.* 32%; OR = 0.63 (0.49-0.82); $p = 0.0006$], marginally less infection related mortality [3.1% *vs.* 5.7%; OR = 0.51 (0.26-1.00); $p = 0.05$] and no significant difference in overall mortality [5.1% *vs.* 7.1%; OR = 0.68 (0.43-1.06); $p = 0.10$] (*2*). Bone, joint pain, and arthralgias were more common in CSF treated patients ($p = 0.007$).

Studies have reported that hypotension and bacteremia in the setting of neutropenia are significant risk factors for prolonged hospitalization (> 7 days) and high mortality. Malik *et al.* reported that mortality rate is associated with febrile neutropenia in patients presenting with shock of 82% (*3*). A study from France reported that patients admitted to an ICU with febrile neutropenia experienced a 54% 30-day mortality rate (*4*).

The chemotherapy regimens used in the current study were moderately myelotoxic. Patients treated with highly myelotoxic regimens received primary G-CSF prophylaxis and were not included in this study.

During the study period, our departmental policy of full-dose administration with secondary G-CSF support was limited to patients with solid tumors who were being treated with an intention for cure or for durable remission. This explains the relatively high proportion of patients with lymphoma (42%) and breast cancer (40%). It is noteworthy that a substantial portion of patients with potentially curable histologically aggressive non-Hodgkin's lymphoma (*5*) and breast cancer patients treated with adjuvant chemotherapy (*6*) have reductions and/or delays in dosage, mostly due to neutropenia. Therefore, secondary G-CSF prophylaxis may play an important role in sustaining dose-intensity in patients with these diagnoses.

Controlled trials show beneficial effects of filgrastim on hospitalization, antibiotic use, incidence of neutropenia with fever, and chemotherapy dosing compared with placebo, when started 24 h after the last dose of chemotherapy (*7-9,15*). Delaying the start of filgrastim therapy beyond 72 h has suboptimal effects on hematological recovery and infection-related endpoints (*16,17*). The use of filgrastim following consecutive cycles of chemotherapy appears to confer cumulative benefits in the management of neutropenia associated complications (*7*).

Our data also demonstrate that, following conventional chemotherapy associated with uncomplicated neutropenic fever, the same regimen can be safely given without dose reduction with secondary G-CSF prophylaxis, given 24 h after the last dose of chemotherapy. The incidence of febrile neutropenia during the first cycle of chemotherapy given with filgrastim supported 32/50 (64%) patients which gradually was reduced to 9/50 (18%) in the second cycle, 5/50 (10%) in the third cycle, and 2/50 (4%) in the fourth cycle. Furthermore, there was no evidence of bacterial infection or other serious infection in any of these patients. Importantly, the rate of other dose-limiting toxicities in patients treated with full dose chemotherapy with filgrastim support was very low and included grade 3 mucositis that developed in one patient in the fourth cycle of chemotherapy with G-CSF.

5. Conclusion

G-CSF secondary prophylaxis may be justified in patients who have experienced a previous febrile episode or prolonged neutropenia, for whom the maintenance of dose intensity is important, *e.g.* those with primary breast cancer, advanced hodgkins disease, or intermediate/high-grade non-Hodgkin's lymphoma (NHL). G-CSF should be started 24-hour post chemotherapy at a dose of 5 μg/kg/day. If delayed for more than 72 h, benefits may be lost. G-CSF administration should be continued until the expected nadir has passed and the neutrophil count has recovered into the normal range. Filgrastim administration through repeated cycles of chemotherapy appears to confer cumulative benefits. Our data also show that a policy of full-dose administration of moderately myelotoxic chemotherapy with G-CSF following a prior cycle that was associated with uncomplicated febrile neutropenia is feasible and relatively safe. Thus, we can say that secondary G-CSF prophylaxis may play an important role in sustaining dose-intensity in these patients.

Thus it is now established that the use of G-CSF will significantly reduce the degree and duration of chemotherapy-induced neutropenia with a substantial reduction in infection and associated morbidity. It was also expected that such a rapid recovery of neutrophils would allow chemotherapy to be given on time and at the appropriate dose. This would be accompanied by an improved tumor response. This goal has not been realized but awaits further adequately-sized randomized studies.

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Case Report

Sarcomatous change of hepatocellular carcinoma in a patient undergoing living donor liver transplantation

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Summary **In a 53-year-old male who received a right liver graft from his son, computed tomography 1 week before living donor liver transplantation (LDLT) revealed three hepatocellular carcinoma (HCC) tumors in the liver that met the Milan criteria. Resected specimen revealed four tumors and microscopically, one of four HCC tumors in the resected whole liver comprised a glandular structure with spindle-like cells indicative of a sarcomatous change in HCC. Two hundred and sixty days after LDLT, the patient complained of left meralgia, which was diagnosed as iliac bone metastasis from HCC. Over a period of 3 months, the iliac bone metastasis rapidly enlarged. The tumor aggressively extended into the patient's bone marrow, causing severe pancytopenia. The patient died 371 days after LDLT. This tumor was detected preoperatively by computed tomography but lack of enhancement. These findings indicate that pathologic evaluation of each tumor is a key to predicting an accurate prognosis.**

Keywords: Liver transplantation, sarcomatous, hepatocellular carcinoma

1. Introduction

Living donor liver transplantation (LDLT) is a therapeutic option for hepatocellular carcinoma (HCC) with end-stage liver disease. Milan criteria (*1*) or more expanded criteria (*2*) are used to select patients with HCC for liver transplantation. Tumor differentiation and pathologic features also affect the prognosis of the patients (*3*). Here, we report a case presenting with severe pancytopenia an early recurrence of a sarcomatous change of HCC in a patient undergoing LDLT.

2. Case report

The subject was a 53-year-old male who received a right liver graft from his son. The patient was indicated with liver transplantation for HCC which could not be treated with partial resection due to liver dysfunction. Laboratory data on admission were as follows: hemoglobin, 14.9 g/dL; platelets, $5.4 \times 10^4/mm^3$; total bilirubin, 3.0 mg/dL; serum albumin, 2.8 g/dL; and prothrombin time international ratio, 1.46. His hepatitis C virus (HCV) RNA titer was positive (< 0.5 Meq/mL) and the genotype was 1b. The alpha-fetoprotein and des-γ-carboxy prothrombin levels before transplantation were 323 ng/mL and 64 mAu/mL, respectively. The patient's condition was complicated with hepatopulmonary syndrome. The partial pressure of oxygen in the arterial blood was 55 mmHg under room-air. The blood types of the recipient and donor were identical. There was only one HLA mismatch at the A, B, and DR loci. The T lymphocytotoxic crossmatch test was negative. The patient was diagnosed with three nodules (Segments 4, 8 and 5/6, each) in the liver based on computed tomography, compatible with HCC (Figure 1). Two of the tumors (in Segments 4 and 8, each) were 1 cm in diameter and the other (in Segment 5/6) was 2 cm.

A right liver graft was transplanted as described elsewhere (*4*). The weight of the graft was 684 g, which corresponded to 58% of the standard liver volume (*5*)

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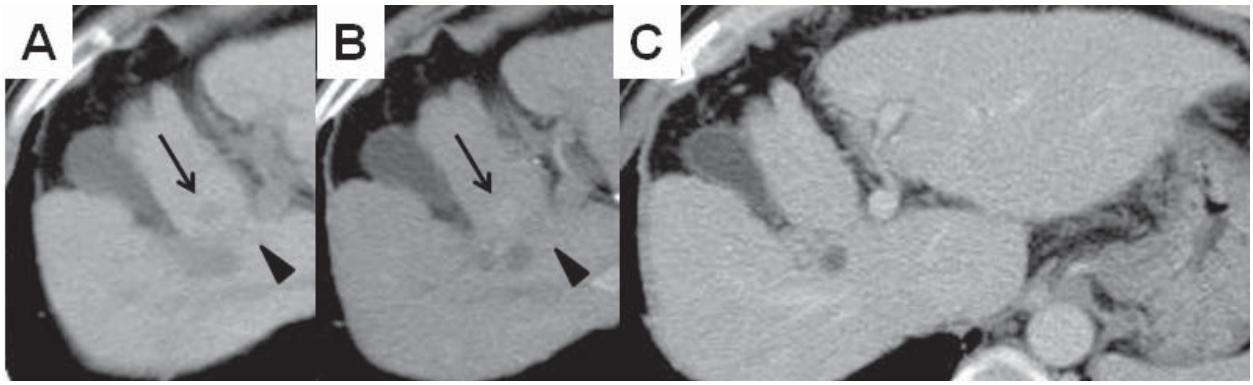

Figure 1. Computed tomography images of the tumor in segment 4. (A) non enhanced; (**B**) early phase; (**C**) late phase. The ventral part (*arrows*) was enhanced in the early phase and in contrast the dorsal part (*arrow heads*) was vague and not enhanced by contrast medium.

Figure 2. Resected specimen. (A) Macroscopic findings of the tumor in S4 (white line indicated 5 cm length). **(B)** The magnified image of **(A)**. The ventral area of the tumor surrounded by arrows was necrotic possibly due to ethanol injection therapy. The dorsal area showed by arrow heads was a viable lesion.

of the recipient. Blood loss during surgery was 4,700 mL. Tacrolimus (Prograf, Astellas Pharmaceutical Corporation, Tokyo, Japan) and methylprednisolone (Solu-Medrol, Pharmacia Corporation, Peapack, NJ, USA) were used as immunosuppressive agents. There were no vascular or biliary complications. He was discharged from our hospital 27 days after LDLT. Two months after LDLT, interferon-alpha 2a (6 MU × 3 per week) and ribavirin (600 mg per day) were administered for HCV infection. His HCV-RNA titer became negative 90 days after LDLT.

There were four grossly visible HCC nodules in the resected whole liver. Besides with the preoperatively diagnosed three tumors, additional tumor was found in segment 1. The size of the largest tumor (segment 5/6) was 1.7 cm in diameter. The tumor, 1.2 cm in diameter in segment 4, showed mixed white tissue and black hemorrhagic foci (Figure 2). Microscopically, it comprised an epithelial component with a pseudoglandular structure and a sarcomatous component with spindle-like tumor cells. The histologic transition between the two components suggested a sarcomatous change in HCC (Figure 3). Immunohistochemical studies supported the diagnosis, as both the sarcomatous component and epithelial component were positive for cytokeratin CAM 5.2, vimentin, and alpha-fetoprotein,

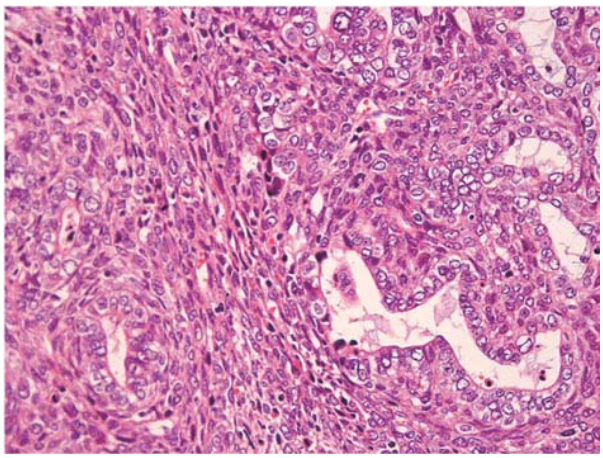

Figure 3. Microscopic findings. This tumor was consisted of glandular structure and spindle like cell.

whereas they were negative for cytokeratin 7, a marker of cholangiocellular carcinoma (Figure 4).

Two hundred and sixty days after LDLT, the patient complained of meralgia in the left leg. Computed tomography findings led to a diagnosis of bilateral iliac bone metastasis of HCC. No other metastatic lesions were detected in the liver graft, lung, brain, or spine. 18-fluoro-2-deoxyglucose positron emission

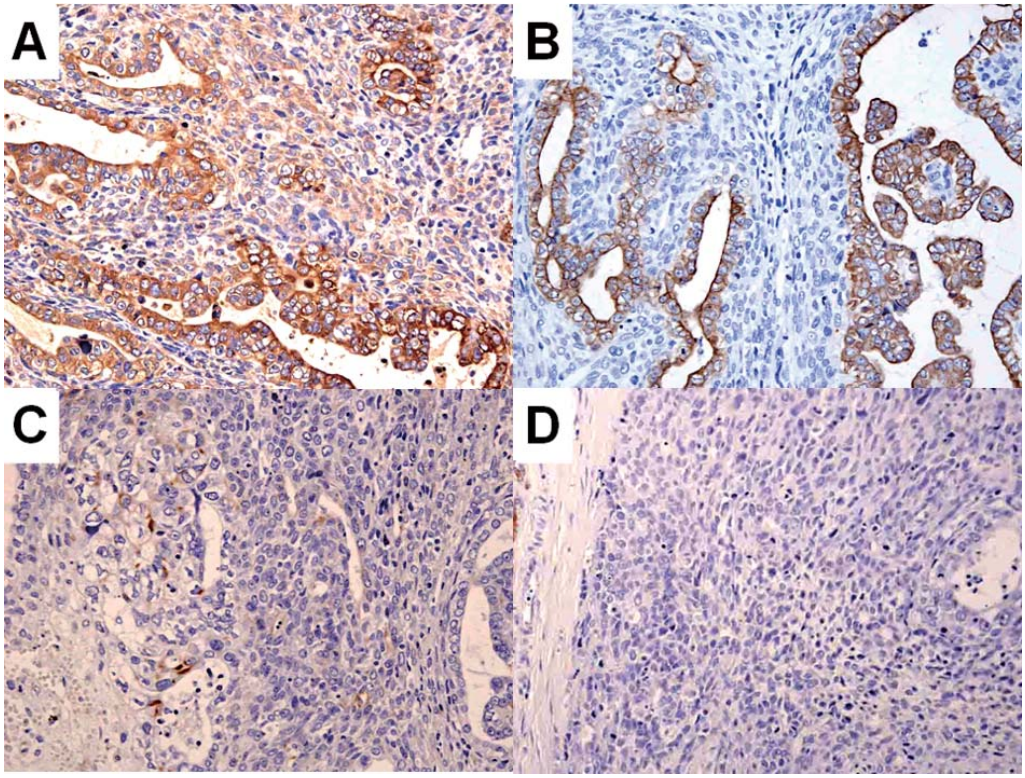

Figure 4. Immunohistochemical study of this tumor. Glandular structure was positive for alpha-fetoprotein **(A)** and cytokeratin CAM 5.2 **(B)**. The spindle like cell was positive for vimentin **(C)** whereas cytokeratin 7 **(D)** was negative.

tomography images, however, showed positive signals in sternum, spine, and iliac bone. The metastatic lesion in the iliac bones rapidly enlarged from 2 cm to 20 cm in diameter over a 3-month period. The periphery of this lesion was well enhanced in the arterial phase on computed tomography. Technetium-99m hydroxymethylene diphosphonate bone scintigraphy showed no uptake in this lesion at diagnosis. He became pancytopenic (white blood cell, red blood cell, and platelet count had decreased to 1,700/μL, 4.8 g/dL, and 0.5×10^4/μL, respectively) 320 days after LDLT. Bone-marrow biopsy did not reveal hematopoietic cells, but there were several conglomerates of atypical cells with high nuclear pleomorphism and scanty cytoplasm. These immature cells could not be diagnosed as metastatic lesions. The patient died 371 days after LDLT. His alpha-fetoprotein and des-γ-carboxy prothrombin levels were 1 ng/mL and 118 mAu/mL, respectively, at that time.

3. Discussion

The coexistence of a sarcomatous component and ordinal HCC is a histologic type of HCC (*6*). Ishak and colleagues (*7*) classified spindle cell (pseudosarcomatous or sarcomatoid) -type HCC as an HCC types in a working group sponsored by the World Health Organization. Several reports indicate an incidence of sarcomatoid HCC in 1.8% of surgically resected cases (*8*) and 3.9% of autopsy cases (*9*). Kakizoe and colleagues (*9*) reported that the sarcomatous component of HCC is derived from a dedifferentiation of anaplasic changes in ordinal HCC rather than collided double cancer.

Nishi and colleagues (*10*) reported that sarcomatoid HCC patients have a poorer prognosis than patients with ordinal type HCC. Hwang and colleagues (*3*) reported the prognosis in 19 patients with sarcomatoid HCC, 15 underwent liver resection and 4 received transplantation. The 3-year survival rate of the 15 patients that underwent liver resection was 18%, although they were judged to be less than stage III according to tumor-node metastasis staging system. Three of the four patients that underwent liver transplantation met the Milan criteria. Their 3-year survival rate was 38%.

Transcatheter arterial chemoembolization and/or percutaneous ethanol injection therapy might accelerate sarcomatous changes of HCC through necrosis and degeneration. Kojiro and colleagues (*11*) reported the results of autopsy series study indicating that 21% of patients who underwent arterial chemoembolization had sarcomatous HCC compared with 4.2% of the patients who did not undergo arterial chemoembolization. Komada and colleagues (*12*) reported a patient with sarcomatous HCC who received several percutaneous ethanol injections over 5 years. The present patient underwent percutaneous ethanol injection therapy 9 times in 6 years before transplantation.

Immunohistochemical studies are important for the

diagnosis of sarcomatous HCC. Kakizoe and colleagues (*9*) reported that 64% of sarcomatous HCC is positive for vimentin. Maeda and colleagues (*8*) reported that 62% of sarcomatous HCC is positive for cytokeratin CAM 5.2. Our present case was positive for both vimentin and cytokeratin CAM 5.2.

Honda and colleagues (*13*) reported that in the delayed enhancement phase of computed tomography sarcomatous HCC appears as an irregular intrahepatic mass, although Hwang did not describe this feature in his series (*3*). Koo and colleagues (*14*) reported that a solid component indicates variable enhancement during three-phase dynamic CT imaging. The present case, however, did not show those features. Hwang (*3*) reported that they diagnosed existing sarcomatous HCC by liver biopsy prior to liver resection surgery.

At our institution, 97 patients have undergone LDLT for HCC with end-stage liver disease in 12 years. This present case is the first case in our series with sarcomatous HCC. The present case indicates that pathologic evaluation of each tumor is key for predicting an accurate prognosis. Liver transplantation for sarcomatous HCC may be contraindicated due to its aggressive features.

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Acknowledgments. All funding sources should be credited in the Acknowledgments section. In addition, people who contributed to the work but who do not fit the criteria for authors should be listed along with their contributions.

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193:149-154.

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